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VOL. 35 MARCH, 1950 No. 3

MAR 30 1950

The Journal of Laboratory and Clinical Medicine

The Official Publication of the Central Society for Clinical Research

Clinical and Experimental

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Vol. 35, No. 3, March, 1950. The Journal of Laboratory and Clinical Medicine is published monthly by The C. V. Mosby Company, 3207 Washington Blvd., St. Louis (3), Mo. Subscription Price: United States, its possessions, Pan-American Countries, $10.00 per year; Canada, $11.00; Foreign, $11.00. Subscriptions to medical students, interns and residents, half price. Entered as Second-Class Matter, October 8, 1915, at the Post Office at St. Louis, Missouri, under Act of March 3, 1879. Printed in the U. S. A.

RATES OF TURNOVER AND BIOLOGIC DECAY OF CHLORIDE AND CHLORIDE SPACE IN THE DOG DETERMINED WITH THE LONG-LIFE ISOTOPE, Cl^{36}

G. E. Burch, M.D., S. A. Threefoot, M.D., and C. T. Ray, M.D.
New Orleans, La.

IN THE past, tracer studies of chloride in biologic experiments have been confined to the use of Cl^{38}, but because of its rapid rate of physical decay ($T^1/_2 \simeq 37$ minutes), its use has been limited to acute experiments. Recently, Cl^{36} ($T^1/_2 \simeq 2 \times 10^6$ years) became available from the Oak Ridge National Laboratories. Although behavior of this radioelement in man is of primary interest, its use in human subjects has been prohibited by the Atomic Energy Commission, pending accumulation of data concerning its rate of turnover in experimental animals. The material, therefore, has been employed to observe the rate of turnover in six dogs studied under controlled metabolic conditions. Because the observations are entirely new and are concerned with data previously unavailable and because of the implications of electrolyte metabolism, edematous states, chloride balance, rate of renal excretion of chloride, rate of exchange of chloride in the blood plasma and also because of the importance in atomic warfare, these investigations are reported.

METHOD

Six young dogs were quarantined for several days and then transferred to separate metabolic cages. Five dogs were normal; one (No. 7012) was found to have amebiasis and was observed for only three days. For three days before the experiment was begun, the dogs were allowed to become stabilized and to establish metabolic equilibrium on the same diet employed during the experiment.*

Each dog was then anesthetized with an intravenous injection of pentobarbital sodium. A cannula was inserted into a jugular vein, clotting of the cannula being prevented by periodic moistening of the stilette with heparin. Cl^{36}, as NaCl, in a dose of 1,000,000 counts per minute per 10 pounds of body weight and in approximately 2 c.c. of water, was injected in an average of 11 seconds (6 to 23) into a vein of a hind leg. The solution contained approximately 18.0 mg. of NaCl. Samples of 1 c.c. of blood were collected from the jugular vein every 5 to 15 seconds for 2 minutes, every 30 seconds for the next 3 minutes, every 1 to 5 minutes for the next 20 minutes, and then every 10 minutes for the remainder of the 60 minutes of the study. Any feces or urine excreted during the period was also collected.

From the Department of Medicine, Tulane University School of Medicine and Charity Hospital of Louisiana.

Aided by grants from the Life Insurance Medical Research Fund, a War Department Grant No. W-49-007-MD389, and the Mrs. E. J. Caire Fund for Research in Heart Disease.

Presented before the Twenty-second Annual Meeting of the Central Society for Clinical Research, Chicago, Nov. 4 and 5, 1949. (J. Lab. & Clin. Med. 34: 1589, 1949.)

Received for publication, Nov. 10, 1949.

*Pard, a product of Swift and Company. It varies in chloride content from 0.24 to 0.48 per cent by weight, the average being 0.36 per cent. Therefore, 1.63 Gm. of chloride per pound (454 Gm.) of food was employed as the intake of chloride.

The dog was then returned to his cage. Urine and feces were collected every morning. The bottom pan in the cage was rinsed twice, this having been found by experiment to be sufficient washing, and the washings were studied for radioactivity. The dog was then weighed. Food and water remaining from the previous 24 hours were measured and another known quantity of each was placed in the cage. A sample of blood was collected on alternate days. Collections of urine and blood were continued until the radioactivity of both reached background.

The volume of the urine and rinsing water and weight of the feces were recorded. Aliquots of the urine, rinsing waters, Formalin suspension of the feces, and blood serum were counted by means of thin mica window Geiger counters.*

After necessary considerations were given to background, volume of the samples, weight of the dogs, and injected dose, it was possible to express the concentration of Cl^{36} in counts per minute per cubic centimeter in terms of an injected dose of 1,000,000 counts per minute per 10 pounds of body weight. It was also possible to express the data in terms of total counts per minute of Cl^{36} excreted in the urine and feces.

RESULTS

(1) Early Concentration-Time Course of Cl^{36} in the Blood Serum.—From the blood sera collected during the short intervals of time after injection of the tracer it was possible to record the rapid decline of concentration of Cl^{36} occurring during the first 60 minutes. The results are summarized by Fig. 1. This curve was analyzed by methods previously described.[1, 2, 3] The individual curves are not shown, since they did not differ significantly from the average curve (Fig. 1).

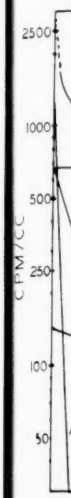
The curve of concentration-time course was found to be composed of at least four major exponential regression rates expressed by the equation

$$CN_t = 1250e^{-2.079t} + 680e^{-0.308t} + 144e^{-0.0377t} + 674e^{-0.000175t},$$

where CN_t is the concentration of Cl^{36} in counts per minute per cubic centimeter of serum at any time,
t represents time in minutes after injection of the tracer, and
e is the base of the natural log.

Comment: The concentration-time course for the Cl^{36} in serum is similar to that reported by Gellhorn and associates[4] in dogs for Na^{24} as well as that reported by Cowie, Flexner, and Wilde[5] for Cl^{38} in guinea pigs. As stated previously, the regression curves represent complex biologic phenomena which are expressions of chemical and physical processes too complicated to interpret properly at this time. The curves have an unlimited number of exponential components, but the four major regression rates are employed for simplification. The initial one is concerned to a large extent with mechanical mixing in the blood stream immediately following the injections. The other regression rates are related to movement of Cl^{36} from the blood serum into compartments at different rates, for example, erythrocytes, interstitial fluid, chambers of the

*Neutron bombardment of KCl results in the production of K^{42} and S^{35} in addition to Cl^{36}. The material remained in this laboratory for several months to insure physical decay of K^{42} ($T_{1/2}$ 12.4 hr.). Although the material had been processed at Oak Ridge National Laboratories to remove S^{35} ($T_{1/2}$ 88 days), the purity was checked by decay curves and Feather's curve. All tests indicated that Cl^{36} was the only radioactive isotope injected into the dogs. As an additional precaution, all samples were counted with the use of an aluminum filter 10.335 mg. per square centimeter to eliminate any S^{35} which may have been present.



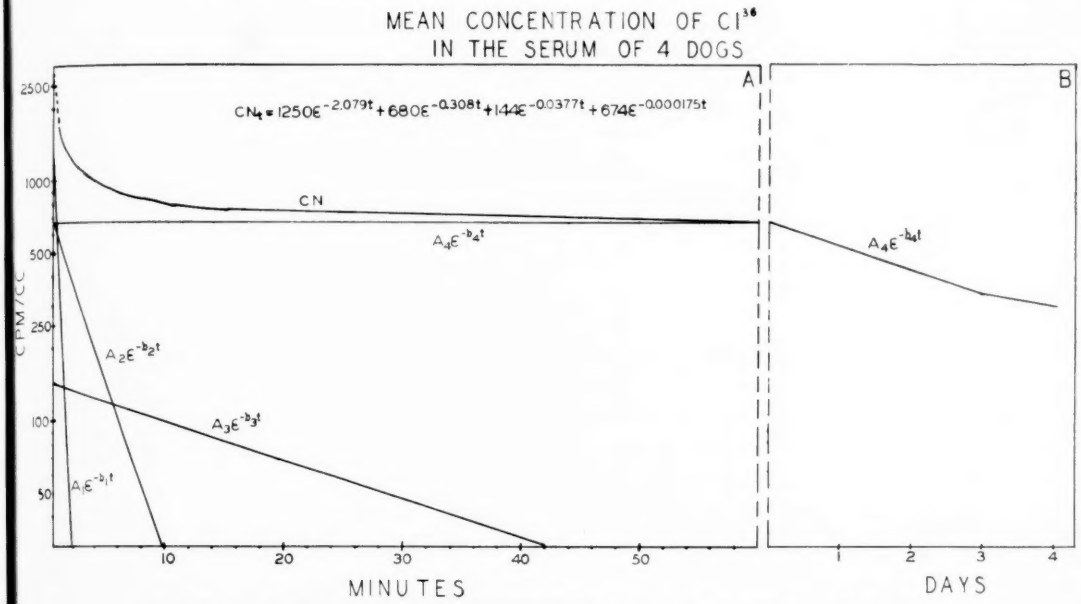


Fig. 1.—*A*, The mean curve of concentration-time course of Cl^{36} for the serum of four dogs for 60 minutes analyzed into 4 regression rates.
B, The mean curve of concentration-time course of Cl^{36} for the serum of the same four dogs for the first 4 days of the experiments. The rate of regression in concentration is identical with the slowest rate of part *A* of this figure.

eye, spinal fluid, and bone, as well as urine and feces. These problems, previously discussed in detail,[2, 3] are much the same for almost all such tracer studies.

Studies on rats and rabbits by Manery and Haege[6] indicated that Cl^{38} penetrated a space equivalent to that of sodium in one hour but that it continued at a slower rate to penetrate certain tissues, especially the brain. Winkler and associates[7] studied the decay of Cl^{38} in blood serum of dogs and considered equilibrium to be established in about one hour. This observation was corroborated in our experiments, the mean curve of the concentration-time course approaching a straight line by the end of 60 minutes (Fig. 1). From the individual curves of the five dogs (Fig. 2), it is evident that the early regression curves decline precipitously. Furthermore, the fourth exponential shown in the equation for the regression curve of Fig. 1 represents excretion primarily. This is evident from the fact that the curves of regression in concentration of Cl^{36} in the serum and of the Cl^{36} mass in the body as a whole are practically parallel, particularly during the first few days before cumulative errors in collection are of consequence and before the counts diminish to a range in which errors in counting are significant (Fig. 2). Another observation supporting the existence of equilibrium at the end of one hour is the fact that the serum concentration for one hour calculated from the slow regression rate was within a few counts of that actually observed from the study of rapid regression in serum concentration at the end of 60 minutes. Therefore, for practical purposes, in the normal dog the equilibrium of distribution is reached relatively quickly.

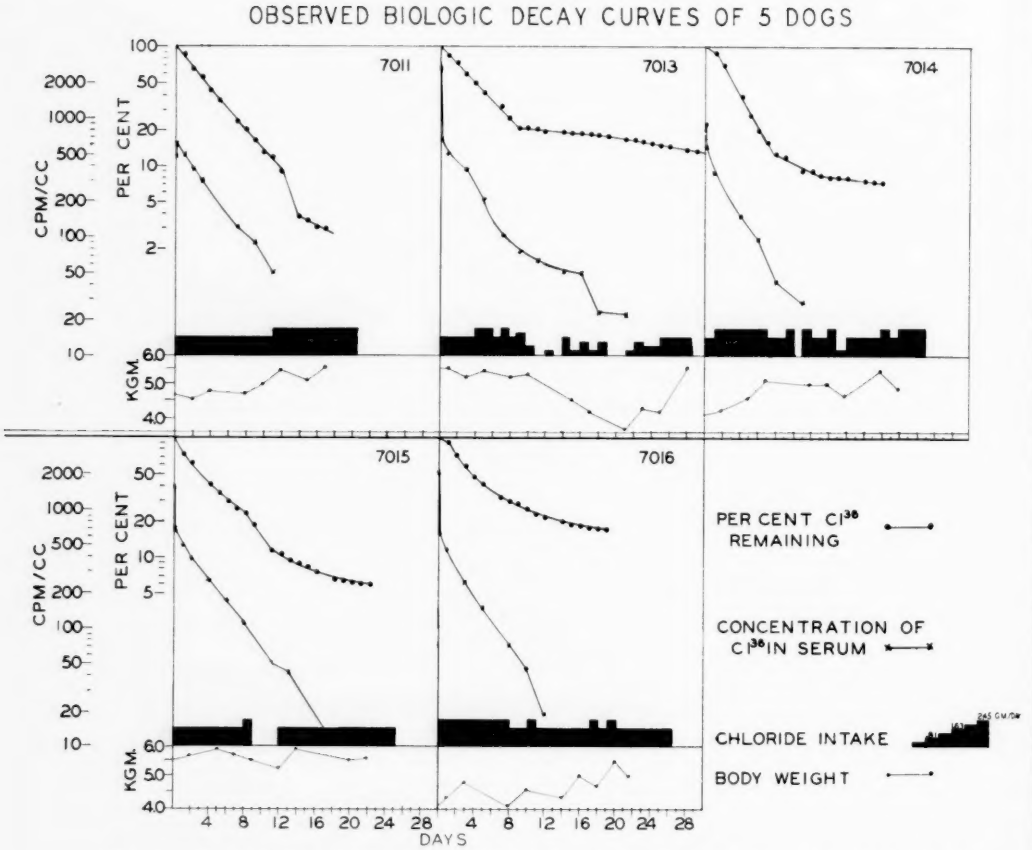


Fig. 2.—The biologic decay rates of Cl^{36} for five dogs. The percentage remaining in the body, dosage minus that accounted for in feces and urine, is calculated as

$$\%N_t = \left(1 - \frac{\sum_{s=1}^{t} k_s}{N_o}\right) \times 100, \text{ where}$$

N_o = injected Cl^{36} in counts per minute; k_s = Cl^{36} excreted in urine and feces during the s^{th} day after injection, expressed in counts per minute; $\%N_t$ = the percentage of injected Cl^{36} not excreted by the end of the t^{th} day after injection. The daily variations in body weight and intake of chloride are shown.

It should be remembered that the term "equilibrium" is employed to indicate equilibrium of distribution. The physiologic problem of steady state is a different one, the distinction becoming evident when comparisons of the regression curves of Figs. 1 and 2 are made.

(2) Rates of Turnover of Chloride Between the Blood Serum and Extraseral Compartments.—From the mathematical approach described by Merrell and associates[1] and employed previously for Na^{24} studies in man,[2] the proportion of serum chloride leaving the blood serum per minute and the proportion of extravascular chloride returning to the blood serum per minute were calculated. The proportion of the total chloride not in the blood serum, as well as that in the blood serum, was also calculated.

Briefly, the approach to the calculations was as follows: If an equilibrium for the curve of the concentration-time course shown in Fig. 1 is assumed to have been reached at the end of about 60 minutes and if mechanical mixing in the serum is considered to be complete at t_o, and since from the general equation of concentration-time course

$$CN_t = A_1e^{-b_1t} + A_2e^{-b_2t} + A_3e^{-b_3t} + A_4e^{-b_4t}, \quad (1)$$

therefore

$$R_s = \frac{A_1b_1 + A_2b_2 + A_3b_3 + A_4b_4}{A_1 + A_2 + A_3 + A_4}, \quad (2)$$

where R_s represents the proportion of serum Cl^{36} leaving the serum per unit of time (one minute). This equation holds since in Equation (1) the coefficients A_1, A_2, A_3, and A_4 represent the relative sizes of the compartments of the Cl^{36} in the serum from which Cl^{36} is leaving, the exponents b_1, b_2, b_3, and b_4 represent the fractions of the respective compartments which leave the serum per unit of time (one minute), and the sum of the coefficients ($A_1 + A_2 + A_3 + A_4$) represents the serum concentration at t_o if equilibrium of distribution of the isotope had been achieved at that time.

$$\text{Therefore, } q = 1 - \frac{A_4}{A_1 + A_2 + A_3 + A_4}, \quad (3)$$

where $\frac{A_4}{A_1 + A_2 + A_3 + A_4}$ is the proportion of total Cl^{36} which is in the serum, and

q is the proportion of total Cl^{36} in the chloride space other than the blood serum.

Then the proportion of Cl^{36} outside the serum which is returning to the serum per unit of time (one minute), R_e, may be represented by the equation

$$R_e = R_s\left(\frac{1 - q}{q}\right). \quad (4)$$

The mean values for the four dogs are shown in Table I. Since Cl^{36} labels the Cl^{35}, then it is evident from Table I that about 75 per cent of the total body chloride was outside the blood serum. The entire chloride of the serum was turned over or exchanged in about one minute. The data also show that about one-third of the chloride outside the serum returned to the serum each minute and about one-fourth moved out of the serum each minute, or about one-half of the entire chloride of the body passed into and out of the serum per minute. Therefore, if a dog weighing 10 pounds contains 5.5 Gm. of chloride, then 2.75 Gm. chloride passed into or out of his serum per minute, which is equivalent to an exchange of 165 Gm. of chloride per hour or 3,960 Gm. Cl or 6,600 Gm. NaCl per day. Thus, the mass of NaCl passing in and out of the serum daily is about 1.2 times the weight of the dog. These quantities would be larger if the chloride mass were larger, as the data in these experiments suggest.

TABLE I. MEAN VALUES OF DISTRIBUTION AND TURNOVER OF CHLORIDE IN BLOOD SERUM AND EXTRACELLULAR COMPARTMENTS FOR FOUR DOGS
(DOGS 7012, 7013, 7015, AND 7016)

q = Proportion of total chloride not in the serum	0.7547
1 - q = Proportion of total chloride in the serum	0.2453
R_s = Proportion of seral chloride leaving the serum per minute	1.02
R_e = Proportion of nonseral chloride returning to the serum per minute	0.3315
$R_s \times (1 - q) = R_e (q)$ = Proportion of total body chloride moving in *or* out of the serum per minute	0.2502
$R_e + R_s$ = Proportion of total body chloride moving in *and* out of the serum per minute	0.5004

Comment: The rate of exchange of Cl^{36} and of Cl^{35} as calculated is based upon the *assumption* that the influence of mechanical mixing in the blood stream is completed early, within about one minute, and therefore contributes relatively little to the initial rapid rate of regression. This assumption, obviously, is not entirely valid. Nevertheless, the calculations were deemed interesting and justifiable since the studies of others[8] would suggest that "transfer" of the freely diffusible ions, such as Na, Cl, or K, from the blood stream through the walls of the capillary endothelium is so rapid that mechanical mixing is the limiting factor. In other words, the rate with which the electrolyte is brought to the capillaries where "transfer" can occur determines the rate of "transfer." This concept is probably true, for the surface area of the capillaries of man is about 6,000 square meters over which flow only about 3 liters of plasma several micra thick. It is, therefore, most probable, if similar anatomic and physiologic comparisons exist in the dog, that the rates of turnover for the dog are of the order described, although the absolute values certainly are subject to error.

The rapid rate with which the tracer reaches equilibrium of distribution throughout the body is consistent with the rapid rates of diffusion across the walls of the capillaries. The significance of these rapid rates of turnover of chloride in electrolyte metabolism and abnormal states of electrolyte and water metabolism, such as congestive heart failure and generalized edematous states, remains unknown and subject almost entirely to conjecture at this time.

(3) Biologic Decay Rates.—The biologic decay rates observed in five dogs are summarized by Table II and Fig. 2. The terminology employed has been described previously[9] and is briefly as follows:

(a) $C_{½}$ represents the length of time required for the concentration of the isotope to reach one-half its value after equilibrium of distribution of the isotope has been reached. The mean $C_{½}$ value for the serum was found to be 2.51 days, with extremes of 2.16 and 3.00 days.

(b) $U_{½}$ represents the length of time required for one-half the injected tracer to be excreted in the urine. The mean $U_{½}$ was found to be 3.50 days, the extremes being 2.75 and 4.00 days.

(c) $E_{½}$ represents the length of time required for excretion of one-half the injected tracer, by all routes, i.e., urine, feces, saliva, tears, and so forth. The mean $E_{½}$ was found to be 3.40 days, the limits being 2.75 and 3.80 days.

TABLE II. CERTAIN OBSERVED BIOLOGIC DECAY PERIODS AND PERCENTAGE RECOVERY OF Cl^{36} IN FIVE DOGS

DOG	$C_{1/2}$ (DAYS)	$U_{1/2}$ (DAYS)	$E_{1/2}$ (DAYS)	BACKGROUND OBTAINED		Cl^{36} RECOVERED		
				SERUM (DAYS)	URINE (DAYS)	URINE (%)	FECES (%)	TOTAL (%)
7011	3.00	3.33	3.33	16.0	18.0	95.51	1.38	96.89
7013	2.50	4.00	3.80	30.0	31.0	81.16	5.89	87.05
7014	2.50	2.75	2.75	15.0	21.0	89.32	3.51	92.83
7015	2.40	3.80	3.50	22.0	21.0	92.48	1.72	94.20
7016	2.16	3.66	3.66	20.0	20.0	82.87	.60	83.47
Mean	2.51	3.50	3.40	20.6	22.2	88.27	2.62	90.89
Max.	3.00	4.00	3.80	30.0	31.0	95.51	5.89	96.89
Min.	2.16	2.75	2.75	15.0	18.0	81.16	.60	83.47

Thus in the normal dog on the diets indicated (Fig. 2) one-half the chloride of the entire body is renewed about every three and one-half days.

Obviously, it is not possible to determine with absolute accuracy the time when all of the tracer is excreted or when all has been collected, because the excretion is exponential in nature and when low concentration of the isotope is reached and the background levels are approached, large errors are introduced. Nevertheless, it was interesting to note the length of time required for the counts in the serum to reach background (Table II), the mean being 20.6 days, with extremes of 15 and 30 days. The mean for the urine was found to be 22.2 days and the extremes were 18 and 31 days. These time values are essentially seven to eight times the $C_{1/2}$ and $E_{1/2}$ values, a quantitative relationship to be expected in an exponential phenomenon with absolute values of such order.

For reasons similar to those just mentioned, the percentage of the injected isotope which was recovered in the excreta is subject to error. Nevertheless, the mean total recovery was 90.8 per cent of the amount injected, the extremes being 82.9 and 96.9 per cent.

Comment: The rapid rate of renewal of chloride in the body of the dog indicates the dynamic nature of the Cl ion in its body. This dynamic state of the chloride ion is supported by the rapid exchange between the chloride compartments of the body, the rapid rate with which equilibrium of distribution is established, and the rapid turnover of chloride in the body as a whole.

Reasons for differences in the $C_{1/2}$ and $E_{1/2}$ values are unknown. They may be due in part to a small daily loss in Cl^{36} during collection, measuring, cleaning the cages, and other such procedures, as well as possible gradual fixation of the Cl^{36} in compartments with relatively slow rates of exchange. Although such compartments may exist, definite ones are not known. It is possible that the rate of turnover of chloride in bone may be as slow as it is for sodium.

The significance of the rapid rate of turnover of chloride in the body and, of course, of the radioactive form can be appreciated in other biologic problems, such as planning of tracer studies with chloride or bromide, metabolic balance studies, bromide poisoning and its therapy, bromide therapy, atomic warfare, and exposure to radiochloride and bromide. These and other implications become evident with consideration of the biologic applications of the observations.

The influence of intake of chloride, change in size of compartment, and other factors upon $U_{\frac{1}{2}}$, $E_{\frac{1}{2}}$, and $C_{\frac{1}{2}}$ are discussed later. For more detailed information reference may be made to similar discussions applied to Na^{22} studies in man.[9, 10]

These tracer studies corroborate data already available which show that only a small amount of chloride escapes from the body in the normal stool. The excretion of the Cl^{36} represented 2.6 per cent (range 0.6 to 5.9 per cent) of the total tracer administered. The difference between the values for $U_{\frac{1}{2}}$ and $E_{\frac{1}{2}}$ is due to that excreted in the feces. These studies also demonstrate the protective role played by the intestinal tract over the electrolyte stores of the body. On the basis of such findings many physiologists believe it permissible to ignore a study of the feces in certain types of metabolic studies of electrolytes.

(4) Chloride Space.—The chloride space was estimated from the regression curves shown in Fig. 2. The data indicate that equilibrium of distribution of the tracer is reached by the end of the first day and almost completely by the end of the first hour. The concentration of the isotope, CN_o, in the serum at t_o was, therefore, determined by extrapolating the curves of serum concentration (Fig. 2) to t_o. This value was then divided into the injected dose N_o for the dog. The resultant is the "serum equivalents" of chloride space in cubic centimeters. The mean chloride space in "serum equivalents" was found to be 34.6 per cent of the body weight of the dog, the extremes being 32.4 and 38.0 per cent (Table III).

TABLE III. CALCULATION OF Cl^{36} "SPACE" IN "SERUM EQUIVALENTS" IN FIVE DOGS*

DOG	AMOUNT INJECTED N_o (CPM)	INITIAL CONCENTRATION CN_o (CPM/C.C.)	Cl^{36} SPACE N_o/CN_o (C.C.)	BODY WEIGHT (GM.)	Cl^{36} SPACE AS % BODY WEIGHT
7011	1,025,000	620	1653	4654	35.52
7013	1,225,000	670	1828	5562	32.87
7014	900,000	580	1552	4086	37.98
7015	1,200,000	680	1765	5448	32.40
7016	900,000	640	1406	4086	34.41
Mean	1,050,000	638	1641	4767	34.64
Max.	1,225,000	680	1828	5562	37.98
Min.	900,000	580	1406	4086	32.40

*By use of the extrapolated value for the initial concentration (CN_o) of Cl^{36} in the blood and the injected dose (N_o) in counts per minute.

The time-course of the chloride space in "serum equivalents" was studied for one dog, Dog 7011. Corrections were made for the Cl^{36} excreted. Results are shown in Table IV. The average space was 39.6 per cent of the body weight as serum equivalents. The time-course difference between the concentration curve and the excretion curve in Fig. 2 is an index of the time-course of chloride space. When the concentration values approach background, errors in counting increase so that only the portions of the curves representing the first five days of the study should be considered to be accurate enough to warrant serious consideration. Since the curves of concentration and excretion are essentially parallel in their course, chloride space therefore remains essentially constant. The time to time variations are evident

from the curves. Because of errors of an undetermined origin (probably in collection of excreta), the calculated space for the other dogs expanded with time.

TABLE IV. TIME-COURSE OF CHLORIDE SPACE IN "SERUM EQUIVALENT" FOR DOG 7011*

DAY t	AMOUNT Cl^{36} REMAINING N_t (CPM)	SERUM Cl^{36} CONCENTRATION CN_t (CPM/C.C.)	Cl^{36} SPACE N_t/CN_t (C.C.)	BODY WEIGHT (GM.)	Cl^{36} SPACE AS % BODY WEIGHT
0	1,025,000	620	1653	4654	35.52
1	901,000	500	1804	4597	39.24
2	678,476	386	1758	4540	38.72
3	578,047	307	1883	4654	40.46
7	242,451	123	1971	4881	40.38
9	172,641	89	1940	4994	38.85
11	124,576	54	2307	5221	44.19
Mean			1902	4792	39.62

*By use of the concentration in the serum at time t (CN_t) and the amount remaining in the body at time t (N_t).

Comment: The size of the chloride space is expressed in "serum equivalents," i.e., the volume of serum that would be necessary to provide a concentration of CN_0 if all of the radiochloride were in serum. The term "serum equivalents" was used because serum was studied and because the concentration of chloride in serum is approximately the same as that of interstitial fluid in general.

The data obtained indicate that the chloride space is larger than commonly considered, being about 35 per cent of the body weight in "serum equivalents" instead of 20 per cent. However, Winkler and co-workers[7] found it to be 25 per cent and suggested that it might be larger. From these data one of three conclusions may be drawn:

(a) The extracellular fluid space is greater than 20 per cent of body weight.

(b) Chloride is found in other compartments than the extracellular fluid in greater quantities than usually considered.

(c) Both the foregoing statements are true.

It is unlikely that the extracellular fluid space in a normal dog is as great as 35 per cent of the body weight of the animal; there are too many physiologic studies that would not support this. It is possible that the extracellular fluid space is somewhat greater than 20 per cent, since the accuracy of the methods available today for these studies has not been fully established and, therefore, may be subject to greater error than usually recognized.

On the other hand, it is probable that chloride is found in cells and bones to a greater extent than most studies indicate. If the extracellular fluid compartment is 20 per cent of the body weight, then the remaining 80 per cent of the body weight would contain 17.5 mEq. per liter of chloride in order that the total chloride space would be 35 per cent of body weight. This is true if there is homogeneity of distribution of chloride in the "tissues" of the body.

Since there is obviously not homogeneity of distribution of chloride in the cells of the body, some tissues, such as muscle, would undoubtedly contain a much higher concentration and others a somewhat lower concentration. Heilbrunn[11] indicated that by his methods of study muscle contains 41 to 51 mEq. Cl per kilogram instead of 24 to 30 mEq. Cl per kilogram as determined by earlier procedures. Therefore, if the value for extracellular fluid space is assumed to be correct, the actual chloride space would be larger than that reported previously. It is known that chloride is found in fairly high concentration inside certain cells of the body, such as gastric mucosa, erythrocytes, renal tubular epithelium, and glomerulus of the sweat glands. It is conceivable that it may also be found in low concentration in other cells of the body. Although the concentration is low, the total amount would be large because of the large cellular mass.

It is also evident that the measurement of extracellular fluid mass based upon chloride studies is subject to considerable question. Observations must be re-evaluated or, at least, considered cautiously until the size and nature of the chloride space are more definitely established.

Variations in chloride space with change in body weight and aspects of the influence of several factors upon the rates of turnover within that space are discussed later.

GENERAL AND THEORETIC DISCUSSION

From the data presented it is evident that chloride must be intracellular in significant quantities. Therefore, it is not possible to employ measurements of chloride space as commensurate with measurement of extracellular water. If it is assumed that the extracellular water of the body is equivalent to 20 per cent of the body weight, then approximately four-sevenths of the chloride of the body is within and the remainder is outside the extracellular body fluid. The reasons for the failure to account for this remainder are not known, but the rapid rate of diffusion of chloride out of cells may be partially responsible. In addition, it has been found that if filter paper disks to which have been added sodium chloride in water at a pH of 7.3 and labeled with Cl^{36} are placed in an oven at 210° C. for twenty minutes, about 60 per cent of the chloride is lost by volatilization. Details of these studies will be reported elsewhere. Therefore, it is possible that dry ashing procedures employed to measure intracellular as well as total body chloride may result in the loss of chloride by volatilization. Such problems require further investigation before definite conclusions may be drawn.

In addition to the foregoing turnover data, there are certain calculations and theoretic considerations for chloride which can be applied to the dog as were applied previously for sodium in man.[11] For example, in the studies with sodium in man it was shown that the rate of intake of nontracer sodium and consequently the rate of output of sodium was one of the most important factors concerned in determining the rate of excretion of the tracer sodium, Na^{22}. The same was found to be true for the dog. A change in body weight and sodium space in man influenced slightly the rate of excretion and

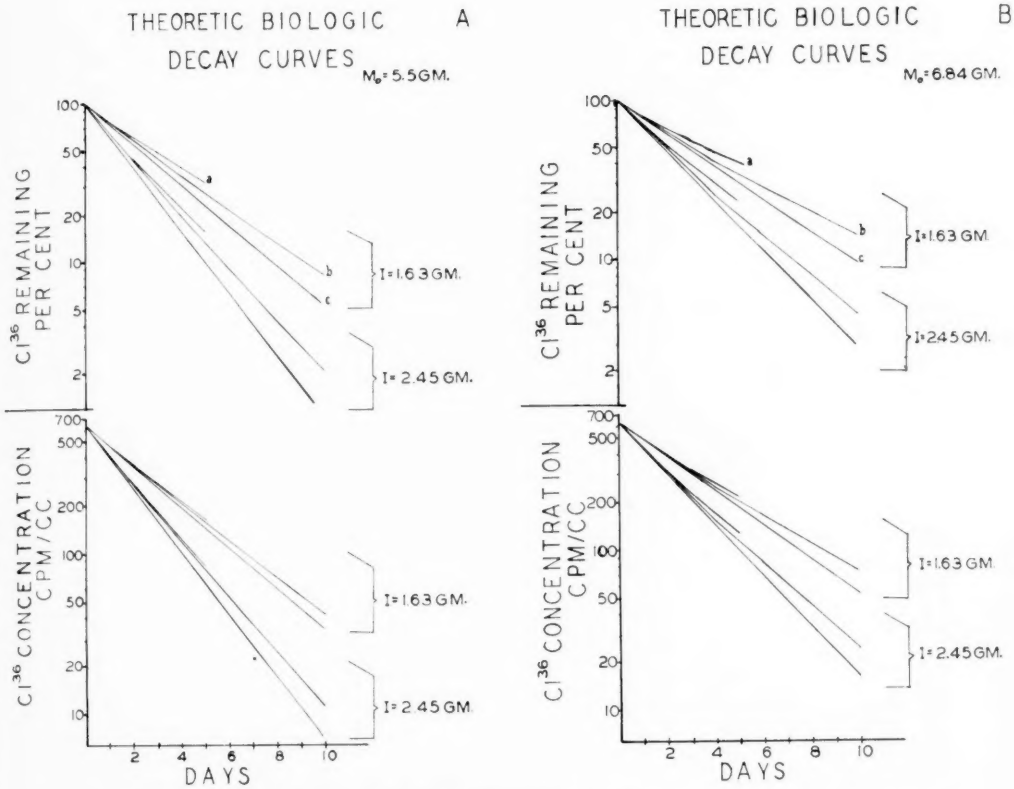


Fig. 3.—*A*, Theoretic curves showing the regressions of Cl^{36} from a dog weighing 4.54 kilograms assumed to have a chloride mass of 5.5 Gm., a value based upon data in the literature. In each group *a* would be the curve if the dog were gaining weight at a rate of 2 pounds in 5 days, *b* the curve if the dog were gaining 2 pounds in 10 days, and *c* if there were no change in weight. *I* is daily intake in grams. M_o is the total body chloride mass.
B, Same scheme as *A* except that the total body chloride mass (M_o) is considered to be 6.84 grams.

concentration of Na^{22} in the blood serum. In the dog, on the other hand, a change in body weight, such as a gain of 2 pounds in five days in a 10 pound dog or a gain of 20 per cent in body weight in five days had little influence on the theoretic rate of decrease in the concentrations of Cl^{36} in the blood serum of the dog (Fig. 3, *A*). This is to be expected since the intake of chloride in the dog is relatively high. A 10 pound dog has, according to the literature (Table V), about 5.5 Gm. of chloride in his body and his intake was 1.63 and 2.45 Gm. a day, which represents 29.6 to 44.5 per cent of the total body chloride. The same type of theoretic analyses is shown in Fig. 3, *B* for a dog with a total chloride mass of 6.84 Gm. per 10 pounds of body weight, a value suggested by the experiments. With this chloride mass a daily dietary intake of 1.63 or 2.45 Gm. represents 23.8 or 35.8 per cent of the chloride mass respectively. It is evident from Fig. 3 that on a similar rate of chloride intake the turnover of Cl^{36} is theoretically more rapid in the presence of the smaller body chloride mass. In the sodium studies the daily intake for man was about 5.0 per cent of the total body sodium. The rate of intake and output of the

TABLE V. TOTAL BODY CHLORIDE FOR A DOG WEIGHING 10 POUNDS (4.54 KG.)*

OBSERVER	ANIMAL	WEIGHT (KG.)	Cl CONCENTRATION (MG./100 C.C. Cl SPACE)	Cl SPACE (C.C.)	CALCULATED TOTAL Cl CONTENT (GM.)	CALCULATED Cl CONTENT OF 4.54 KG. (10 LB.) DOG (GM.)
Winkler and associates[7]	Dog 1	11.0	378	2760	10.43	4.3
	Dog 2	11.4	397	2580	10.24	4.077
Harrison, H. E., Darrow, D. C., and Yarnet, H.: J. Biol. Chem. **113**: 515, 1936	Dog 1	5.81	430.5	1510	6.5	5.079
	Dog 2	6.27	441.0	1756	7.74	5.6
Shohl, A. T.: Am. Chem. Soc. Mon. 82, N. Y., 1939, Reinhold Pub. Corp.	Man	70.0			85.0	5.51
Burch and associates	Mean of 5 dogs	4.54	435	1573	6.84	6.84

*Calculated from data reported in the literature and obtained in these experiments.

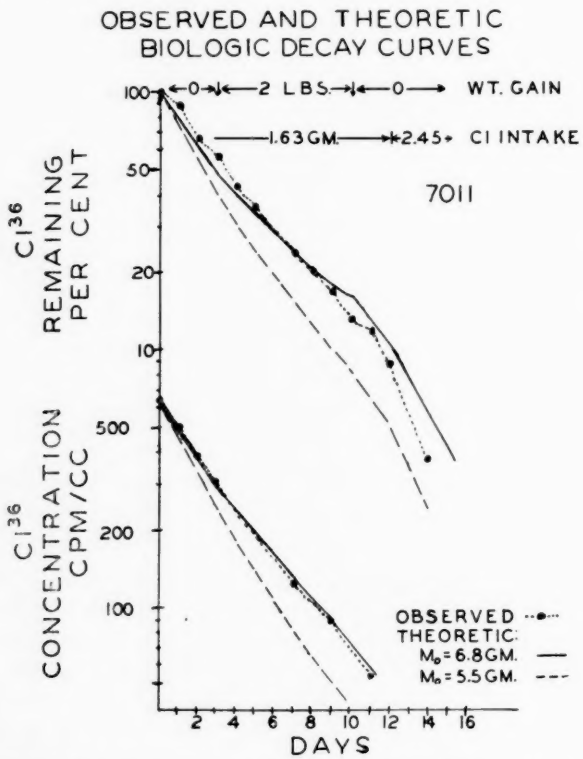


Fig. 4.—Comparison of theoretic with observed biologic decay curves. The dots represent the observed values, the solid line the theoretic curve derived from the observed intake and changes in weight if the chloride mass were 6.84 Gm., and the broken line the same curve if the chloride mass were 5.5 grams.

regular form of the electrolyte is the most influential factor governing turnover of the tracer material.[10] Change in body weight and chloride space are relatively less influential factors than intake in the dog. This is borne out

by Figs. 3 and 4, which show that the theoretic $C_{\frac{1}{2}}$ and the curves of concentration-time course and rate of excretion tend to remain little changed with large percentage changes in body weight of the dogs. The normal average chloride intake for the dog is relatively high, so that a considerable change is necessary to reflect its influence upon the curves.

Comparison of actually observed curves with theoretic ones is shown in Fig. 4. They are remarkably similar where the mass of chloride in the body is assumed to be 6.84 Gm. for the theoretic circumstances. Furthermore, the closer similarity, or almost superimposition, of the observed biologic decay curves to the theoretic one based upon a chloride mass of 6.84 Gm. than to the theoretic curve based upon a chloride mass of 5.5 Gm. is in support of the larger chloride mass as the more correct value for this dog.

The importance of these factors can be appreciated when experiments concerned with electrolyte metabolism in dogs are compared with similar experiments in man. For example, a man weighing 70 kilograms, on a low sodium diet, 200 mg. daily, and with a total body sodium of 60 Gm. is receiving 0.33 per cent of his body sodium in his daily diet. A dog weighing 4.54 kilograms (10 pounds) with a comparable total body sodium would have to receive 12.8 mg. of sodium in his daily diet to be on a comparable diet. This is difficult to achieve and makes it difficult to compare the influence of sodium upon edematous or hypertensive states in the dog with those in man. This may be true for other animals. For even better appreciation of this a comparison may be made of the intake of a normal dog on an average daily intake of sodium chloride of 2.68 or 1.05 Gm. of sodium or 1.63 Gm. of chloride, which represents 29.6 per cent of his body chloride. A man would have to take in about 43 Gm. of sodium chloride a day to be on a diet comparable to that of the dogs. However, a diet low in sodium for a dog usually may be compared only insofar as the dog's daily intake is concerned and not with respect to his total body sodium.

The theoretic considerations previously reported for sodium in man[10] have many other important applications in turnover studies of tracer substances. They are too numerous to permit mention of more than a few in this report but become evident with careful consideration of the equations. For example, three additional applications are:

(1) If there is no gain in size of compartment after equilibrium of distribution has been established and if the exchange is free and dynamic, it is possible to determine the total body chloride also from the equation[10]:

$$CN_t = CN_o e^{-\frac{I}{M_o}t} \quad \text{or} \quad M_o = \frac{I}{-\log_e \frac{CN_t}{CN_o}} \times t.$$

This general equation becomes:

$$M_o = \frac{I}{-\log_e 0.5} \times C_{\frac{1}{2}},$$

where M_o = mass of chloride in the animal's body in grams,

I = daily intake of chloride in grams,

$C_{½}$ = time at which CN_t is 50 per cent of CN_o or the concentration is reduced to 50 per cent of its initial value.

Table VI shows values for the total body chloride obtained from this equation.

TABLE VI. CALCULATION OF TOTAL BODY CHLORIDE*

DOG	$C_{½}$ (DAYS)	INTAKE I (GM./DAY)	TOTAL BODY CHLORIDE		BODY WEIGHT (GM.)	TOTAL BODY CHLORIDE AS % BODY WEIGHT ($\frac{M_o}{BW} \times 100$)	CHLORIDE CONCENTRATION ($\frac{M_o \times 100{,}000}{\text{SPACE}}$) (MG./100 C.C.)
			M_o (GM.)	M_o PER 10 LB. BODY WEIGHT (GM.)			
7011	3.00	1.63	7.05	6.8	4654	.151	426
7013	2.5	1.63	5.88	4.8	5562	.105	322
7014	2.5	2.45	8.84	9.8	4086	.216	570
7015	2.4	1.63	5.6	4.64	5448	.103	317
7016	2.16	2.45	7.6	8.44	4086	.186	541
Mean	2.51	1.96	6.99	6.896	4767	.152	435

*The mass of chloride in grams calculated to be present from the equation $M_o = \frac{I}{(-\log_e 0{,}5)} \times C_{½}$. The estimated concentration in milligrams per 100 c.c. is calculated from this mass of chloride and the Cl^{36} space is recorded for each dog in Table III.

In Table V is shown the total body chloride calculated from the data obtained in these experiments and from the observations of others. A chloride content of 5.5 Gm. in a dog weighing 10 pounds (4.54 kilograms) was chosen arbitrarily from the calculations shown in Tables V and VI.

(2) If the intake and body mass of the regular form of the substance are known, it is possible to predict the $C_{½}$ for a tracer substance from the equation:

$$C_{½} = \frac{M_o\ (-\log_e 0.5)}{I}\ .$$

This equation has many applications in biology and atomic warfare.

Comparisons of $C_{½}$ for dogs and man are presented in Table VII. Values obtained by calculation and experiment show remarkable agreement. This holds if there is no change in the size of the compartment. It is, therefore, possible to predict closely the biologic half-life of such electrolytes as Na and Cl from such equations.

(3) It is also possible from the same general equation to calculate the intake (I) necessary for a given rate of change and total mass of the tracer substance. For example,

$$I = \frac{M_o\ (-\log_e \frac{CN_t}{CN_o})}{t}\ .$$

SUMMARY

1. The concentration-time course in the blood serum and turnover rates within the body in acute experiments and the concentration-time course, time-course of excretion in the urine and feces, and turnover rates for the entire body were studied in six normal dogs observed continuously for eighteen to thirty-one days under controlled metabolic conditions with long-life radiochlorine, Cl^{36} ($t_{½}$ about 2×10^6 years), as a tracer substance.

TABLE VII. COMPARISON OF INTAKES OF SODIUM AND CHLORIDE FOR MAN AND DOGS*

	ANIMAL	WEIGHT (KG.)	$C_{\frac{1}{2}}$ (DAYS)	SODIUM M_o (GM.)	SODIUM I (GM.)	SODIUM $\frac{I}{M_o} \times 100$ (%)	SODIUM NaCl† (GM.)	CHLORIDE M_o‡ (GM.)	CHLORIDE I (GM.)	CHLORIDE $\frac{I}{M_o} \times 100$ (%)	CHLORIDE NaCl† (GM.)
Condition a: Based on rate of biologic decay in normal dog	Man	70	2.51	60	16.57	27.6	42.14	85	23.47	27.61	38.68
	Dog	4.54	(2.51)§	3.89	1.07	27.5	2.72	5.5	(1.63)	27.45	2.48
	Ratio: Man / Dog	15.4		15.4	15.5		15.5	15.5	1.51 15.5		15.6
Condition b: Based on rate of biologic decay in normal man	Man	70	(13.3)	60	(3.1)	(5.2)	(8.0)				
	Dog	4.54	13.3	3.89	3.13 .203	5.22 5.22	7.96 .516	85 5.5	4.43 .286	5.211 5.200	7.3 .471
	Ratio: Man / Dog	15.4		15.4	15.4		15.4	15.5	15.5		15.5
Ratios of Conditions a and b for Man and for Dog			b:a		a:b	a:b	a:b		a:b	a:b	a:b
	Man		5.3		5.3	5.3	5.3		5.3	5.3	5.3
	Dog		5.3		5.3	5.3	5.3		5.3	5.3	5.3

*The intake (I) of sodium and chloride calculated as necessary to produce the $C_{\frac{1}{2}}$ values shown, provided there is no change in size of compartment. The $C_{\frac{1}{2}}$ of 2.51 days is that observed for Cl^{36} in dogs. The $C_{\frac{1}{2}}$ of 13.3 is that observed for Na^{22} in normal man.[7] M_o = The total amount in grams of the element in the animal. $\frac{I}{M_o} \times 100$ = The percentage of the total Cl or Na mass represented by the intake per day.

Where M_o is constant, to shorten $C_{\frac{1}{2}}$ by a factor of 5.3, the intake (I) must be increased by a factor of 5.3. Likewise if M_o is 15.4 times as large for one animal (man) as for another (dog), then the intake (I) must be 15.4 times as large for the $C_{\frac{1}{2}}$ to be equal.

It is evident that the relationship of $\frac{I_a}{I_b} = \frac{C_{\frac{1}{2}b}}{C_{\frac{1}{2}a}}$ holds where I_a is initial intake, I_b is the necessary or new intake, $C_{\frac{1}{2}a}$ is the initial time required to reach one-half concentration, $C_{\frac{1}{2}b}$ is the desired time. Or, in an animal whose compartment size is constant, a desired change in the slope of the biologic decay curves may be obtained by changing the intake by the reciprocal of the ratio of the initial and "desired" slopes. This holds true for both positive and negative changes.

There is remarkably close agreement between calculated and experimentally obtained values.

†The daily NaCl intake required to produce $C_{\frac{1}{2}}$ value indicated.

‡Taken from Shohl, A. T.: Am. Chem. Soc. Mon. 82, New York, 1939, Reinhold Publishing Corporation.

§Values in parentheses were observed.

2. The mean concentration-time course of Cl^{36} in the blood serum for the first 60 minutes after intravenous administration can be expressed by the multiple exponential equation

$$CN_t = 1250e^{-2.079t} + 680e^{-0.308t} + 144e^{-0.0377t} + 674e^{-0.000175t},$$

where CN_t is the concentration of Cl^{36} in the serum at any time t.

3. All the seral chloride leaves the serum each minute and about one-third of the nonseral chloride returns to the serum each minute. Thus, about 2.75 Gm. of chloride pass out of and into the serum each minute or about 3960 Gm. daily, which is equal to 6600 Gm. of NaCl or about 1.2 times the body weight of the dog.

4. The mean $C_{\frac{1}{2}}$ value for the blood serum was 2.51 days (range 2.16 to 3.00 days), the mean $U_{\frac{1}{2}}$ was 3.50 days (range 2.75 to 4.00 days), and the mean $E_{\frac{1}{2}}$ was 3.40 days (range 2.75 to 3.80 days).

5. The mean total recovery was 91 per cent of that administered (range 83 to 97 per cent), 88 per cent (range 81 to 96 per cent) being in the urine and 2.6 per cent (range 0.6 to 5.9 per cent) in the feces.

6. The mean chloride space in "serum equivalents" was 35 per cent of the body weight (range 32 to 38 per cent) and the mean total body chloride was 6.84 Gm. for a dog weighing 10 pounds (4.54 kilograms).

7. These experiments indicate that:

a. The chloride space is larger than previously reported.

b. Chloride space is not a measure of the extracellular fluid space.

c. Chloride is present in considerable quantities outside the extracellular fluid space.

d. A man weighing 70 kilograms would have to consume 43 Gm. of NaCl daily to have a dietary intake of this salt comparable to that of an average dog.

e. A diet of 200 mg. of sodium for a man weighing 70 kilograms is equivalent to a diet of 13 mg. for a dog weighing 4.54 kilograms (10 pounds). Thus the usual studies of low sodium metabolism in small animals with hypertension must be evaluated cautiously. A man of 70 kilograms must consume 15 to 22.5 pounds of food daily to take in equivalent quantities of the same food substances as a normal dog of 4.54 kilograms (10 pounds).

8. The importance of these experiments in the planning of tracer studies with chloride and bromide and understanding metabolism and excretion of chloride and bromide, bromide therapy and intoxication, edematous states, intoxication due to radiochloride and radiobromide in atomic warfare, and other physiologic processes is evident from the data.

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THE EFFECT OF A PERFORATED EARDRUM UPON THE BASAL METABOLIC RATE

JOHN L. SWITZER, M.S., M.D.,* AND LAWRENCE E. GATES
HINES, ILL.

THIS subject is reported, not as a new clinical entity, but to re-emphasize the fact that oxygen leakage from the closed circuit of the basal metabolic apparatus and patient will result in abnormally high results such as those seen in hyperthyroidism, polycythemia, leucemia, diabetes insipidus, and cardiorenal disease with dyspnea.† A review of the literature of the last fifteen years did not reveal a specific publication concerned with this entity.

A perforated eardrum, if the eustachian tube is patent, allows leakage of oxygen, supplied by the apparatus to the pharynx, through the eustachian tube and the middle ear to the external auditory meatus and the atmosphere. Inasmuch as the basal metabolic value is calculated by the volume of oxygen consumed, leakage results in an elevated basal metabolic rate.

This case report illustrates the effect of oxygen leakage from a perforated eardrum which yielded B.M.R. reports equivalent to those seen in hyperthyroidism and their return to normal after oxygen escape was blocked. In addition corroboration of thyroid normaley was obtained by radioactive iodine uptake studies.

METHOD

The technique of obtaining the basal metabolic rate was that delineated in the McKesson Metabolor instruction book. The B.M.R. tracings were obtained by a certified laboratory technician well versed in the methods employed. The technician was not aware of the project undertaken until the series was completed. The basal metabolic rate was obtained after a one-hour rest and with the patient in a fasting state. The patient was unaware of the purpose of the multiple tests.

The tables used for the calculation of the results were the Consolidated Tables for the McKesson Recording Metabolor (Aub and DuBois normal standards) plus the caloric equivalent conversion chart compiled by Boothby and Sandiford (Mayo Clinic). The data obtained with each B.M.R. graph are tabulated in Fig. 1.

CASE REPORT

This 35-year-old white man was admitted to the Veterans Administration Hospital, Hines, Ill., because of dizziness, weakness, nervousness, questionable slight fever, and vague abdominal complaints. Physical examination was negative except for a large central perforation of the left ear drum. There was a small amount of adenoid tissue. There was conduction deafness. No vestibular disease was found. The patient, using the Valsalva maneuver, was able to force cigarette smoke out of his left ear (unequivocal demonstration of eustachian tube patency).

From the Medical Service, Veterans Administration Hospital.

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Received for publication, Oct. 27, 1949.

*Resident in Medicine.

†Best, C. H., and Taylor, N. B.: The Physiological Basis of Medical Practice, ed. 2, Baltimore, 1940, Williams and Wilkins Company, chap. xlvi.

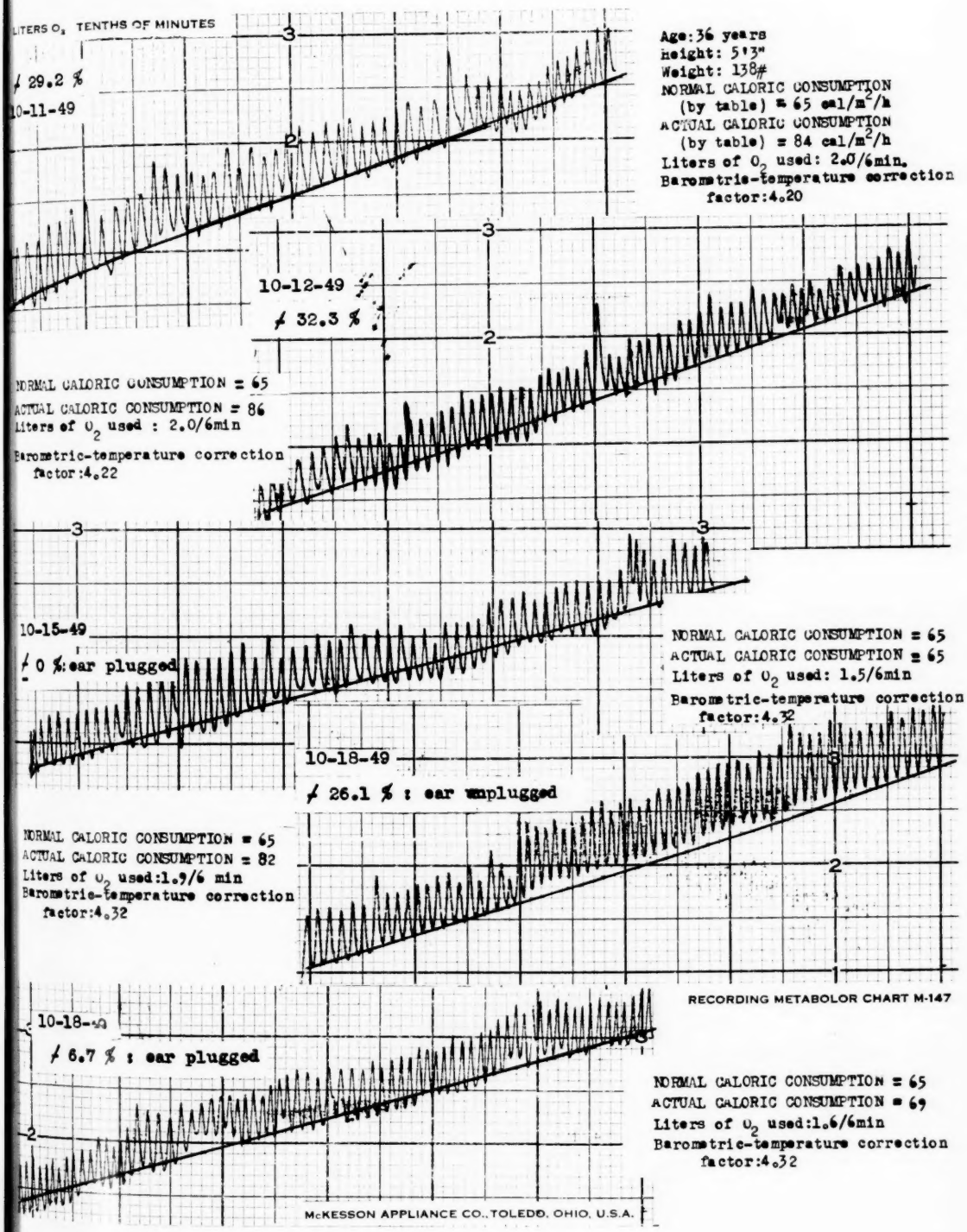

LITERS O₂ TENTHS OF MINUTES
∮ 29.2 %
10-11-49
Age:36 years
Height: 5'3"
Weight: 138#
NORMAL CALORIC CONSUMPTION
(by table) = 65 cal/m²/h
ACTUAL CALORIC CONSUMPTION
(by table) = 84 cal/m²/h
Liters of O₂ used: 2.0/6min.
Barometric-temperature correction
factor:4.20
10-12-49
∮ 32.3 %
NORMAL CALORIC CONSUMPTION = 65
ACTUAL CALORIC CONSUMPTION = 86
Liters of O₂ used : 2.0/6min
Barometric-temperature correction
factor:4.22
10-15-49
∮ 0 %: ear plugged
NORMAL CALORIC CONSUMPTION = 65
ACTUAL CALORIC CONSUMPTION = 65
Liters of O₂ used: 1.5/6min
Barometric-temperature correction
factor:4.32
10-18-49
∮ 26.1 % : ear unplugged
NORMAL CALORIC CONSUMPTION = 65
ACTUAL CALORIC CONSUMPTION = 82
Liters of O₂ used:1.9/6 min
Barometric-temperature correction
factor:4.32
RECORDING METABOLOR CHART M-147
10-18-49
∮ 6.7 % : ear plugged
NORMAL CALORIC CONSUMPTION = 65
ACTUAL CALORIC CONSUMPTION = 69
Liters of O₂ used:1.6/6min
Barometric-temperature correction
factor:4.32
McKESSON APPLIANCE CO., TOLEDO, OHIO, U.S.A.

Fig. 1.

The temperature was never higher than 99.6° F. rectally. An initial B.M.R. was +29.2 per cent. The graph was technically satisfactory. A repeat B.M.R. was +32.3 per cent, and again was satisfactory. The blood cholesterol level, 258 mg. per cent, was not compatible with hyperthyroidism. The blood studies (R.B.C. 5,200,000; Hb. 15 grams; W.B.C. 7,200; polymorphonuclears 59 per cent; lymphocytes 41 per cent; sedimentation rate 6 mm. per hour) were not compatible with polycythemia or leucemia. No cardiorenal or respiratory disease was found. The patient was not edentulous and the rubber mouthpiece of the apparatus fitted snugly, as did the nasal pincers.

The left external auditory meatus was plugged with cotton and sealed with petrolatum; a B.M.R. was obtained and was ± 0 per cent. Inasmuch as these three B.M.R. determinations were done on three separate occasions, repetition of the tests in sequence on one occasion was carried out to obviate the effect of uncontrolled studies. A control B.M.R. with the external meatus patent was obtained, and immediately thereafter the ear was occluded and a second B.M.R. was obtained after a ten-minute interval to insure basal conditions. After technically satisfactory graphs were obtained they were calculated: the initial graph was + 26.1 per cent, the latter, after occluding the left ear, was + 6.7 per cent. (Fig. 1.)

Radioactive iodine uptake studies classified the thyroid as euthyroid. (I^{131} retention: 36.5 per cent in twenty-four hours; protein bound I^{131}: 0 per cent in twenty-four hours.)

SUMMARY

The effect of a perforated eardrum, allowing free passage of oxygen from the closed basal metabolic circuit into the atmosphere, is reported. The absence of abnormal B.M.R. reports following the sealing of the oxygen leak was confirmatory. Normal radioactive iodine uptake studies were further corroboration of the normalcy of this patient's thyroid gland.

CONCLUSION

It is re-emphasized that every patient with abnormal B.M.R. reports should be re-examined in the light of the foregoing data to rule out a [patent] perforated eardrum and a patent eustachian tube.

THE EFFECT OF THYROID- AND OF DINITROPHENOL-INDUCED HYPERMETABOLISM ON PLASMA AND TISSUE LIPIDS AND ATHEROSCLEROSIS IN THE CHOLESTEROL-FED CHICK

J. Stamler, M.D.,* E. N. Silber, M.D., A. J. Miller, M.D., L. Akman, M.D.,†
C. Bolene, B.A.,‡ and L. N. Katz, M.D.
Chicago, Ill.

THYROID hormone inhibits both cholesterol- and stilbestrol-induced atherosclerosis in the chick.[1, 2] It has a similar effect in the cholesterol-fed rabbit.[3, 4, 5] Hypothyroidism plus exhibition of cholesterol is essential for the production of experimental atheroma in dogs.[6, 7] Considerable clinical and experimental evidence exists confirming the interrelationship of thyroid function, lipid metabolism, and atherogenesis.[8-12] Despite a wealth of empiric data, the mechanisms of this interrelationship remain obscure. Thus it is unclear if the influence of thyroid hormone on lipid metabolism and atherogenesis is due simply to hypermetabolism. We undertook to investigate the relation of metabolic rate to experimental cholesterol-induced atherosclerosis by utilizing dinitrophenol, a drug known to cause hypermetabolism.[13-16] This report concerns itself with a comparison of the effects of dinitrophenol and thyroid respectively on lipid metabolism and atherogenesis in cholesterol-fed chicks.

METHODS

One-day-old Hy-Line cockerels were obtained from a commercial hatchery and raised in a battery brooder. They were fed a commercial chick starter mash of known composition.[17] At 8 weeks of age, eighty-four birds were divided into seven groups of twelve birds each. Thereafter they were maintained on the experimental diets indicated in Table I. Essentially, the effects of thyroid and dinitrophenol respectively were studied in experimental birds fed 2 per cent cholesterol mash (Subgroups A, Table I) or 2 per cent cholesterol-5 per cent cottonseed oil mash (Subgroups B, Table I). Appropriate plain mash§ and cholesterol mash controls were maintained (Groups 1 and 4, Table I). Desiccated thyroid‖ was mixed with the various mashes in a concentration of 500 mg. per cent (Group 3, Table I). The dose levels of dinitrophenol (Group 2, Table I) were as follows: First through fifth week, 0.04 per cent; sixth week, 0.05 per cent; seventh and eighth weeks, 0.06 per cent; ninth and tenth weeks, 0.08 per cent; eleventh through thirteenth week, 0.09 per cent. Signs of toxicity were observed on this last dosage level. The dinitrophenol in the diet was therefore cut to 0.07 per cent for the duration of the experiment.

All birds were weighed weekly and a record of feed intake was maintained. They were bled periodically from the alar vein. Pooled aliquots of plasma were analyzed for the various lipid fractions by the methods of Schoenheimer and Sperry[18] and Man and associates.[19, 20] All surviving chicks were sacrificed by decapitation and exsanguination after

From the Cardiovascular Department, Medical Research Institute, Michael Reese Hospital. This Department is supported in part by the Michael Reese Research Foundation.
Aided by a grant from the Life Insurance Medical Research Fund.
Received for publication, Dec. 5, 1949.
*Herbert G. Mayer Memorial Fellow.
†Extra-Mural Fellow in Cardiology, Sinai Hospital, Baltimore, Md.
‡Deborah V. Dauber Memorial research assistant.
§Concurrently we studied the effects of thyroid and dinitrophenol respectively on lipid metabolism and atherogenesis in the chick fed plain mash. These data are being prepared for publication.
‖We are indebted to Dr. L. P. Anderson and Dr. J. R. Mote of the Armour Laboratories, Chicago, Ill., for generous supplies of this hormone preparation.

sixteen to seventeen weeks on the experimental diet. All the viscera were examined and the gross findings recorded. The heart and great vessels (aorta, brachiocephalic and iliac arteries) were carefully inspected for evidence of gross atheromatous plaques. Lesions, if any, were described and recorded graphically on special forms; the aortas were graded 0 to 4 for atheroma, according to criteria previously described.[21] Birds from different groups were autopsied on the same day. The specimens were indiscriminately mixed and graded consecutively as unknowns. Blocks of tissue were taken for microscopic examination and fixed in 10 per cent aqueous Formalin. Frozen sections were stained with sudan IV for lipids and paraffin sections were stained with hematoxylin-eosin. During final sacrifice, pooled aliquots of liver and aorta were analyzed for the lipid fractions according to procedures previously described.[17]

TABLE I. EXPERIMENTAL DIETS USED IN THE SEVERAL GROUPS AND SUBGROUPS

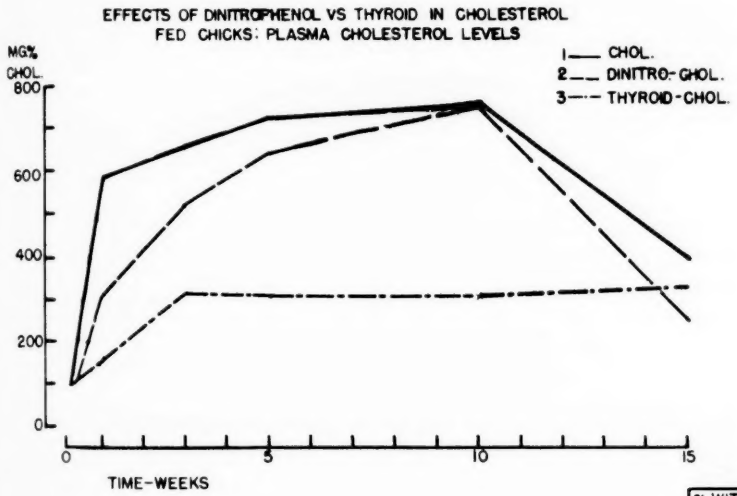
GROUP	SUBGROUP	CHOLESTEROL (%)	COTTONSEED OIL (%)	DESICCATED THYROID (%)	DINITROPHENOL (%)
1	A1	2	0	0	0
	B1	2	5	0	0
2	A2	2	0	0	0.04-0.09
	B2	2	5	0	0.04-0.09
3	A3	2	0	0.5	0
	B3	2	5	0.5	0
4	4	0	0	0	0

RESULTS

Feed Intake, Growth, and Development.—All experimental diets were well tolerated, except for the 0.09 per cent dinitrophenol given during the eleventh through the thirteenth experimental week. After two to two and one-half weeks on this dosage level, birds exhibited decreased feed intake, weight loss, and anemia. A few chicks died. These signs of toxicity necessitated lowering the concentration of dietary dinitrophenol to 0.07 per cent. At this dosage level, dinitrophenol induced no significant consistent alteration in feed consumption or rate of weight gain. Desiccated thyroid (0.5 per cent) was also without significant effect; this dosage is somewhat below the toxic level for the chick.[1] All six groups fed cholesterol exhibited essentially normal feed intake and rate of weight gain.[22]

Plasma and Tissue Lipids.—Corresponding sets of chicks fed cholesterol with or without cottonseed oil exhibited essentially similar plasma and tissue lipid levels. Thus Subgroups A1 and B1 of Group 1 had similar lipid patterns, as did Subgroups A2 and B2 of Group 2 and Subgroups A3 and B3 of Group 3. The incidence, extent, and severity of atherosclerosis were also similar in these paired subgroups receiving similar diets except for the presence or absence of 5 per cent cottonseed oil. Hence we have pooled the data on chicks receiving cholesterol with and without oil. In the following presentation, therefore, Group 1 (A1 plus B1, Table I) is made up of all birds receiving 2 per cent cholesterol mash (with and without cottonseed oil); Group 2 (A2 plus B2), 2 per cent cholesterol plus dinitrophenol mash; Group 3 (A3 plus B3), 2 per cent cholesterol plus thyroid mash; Group 4, plain mash (controls).

The trend of plasma total cholesterol concentrations is presented in Fig. 1. The birds fed cholesterol (Group 1) and cholesterol plus dinitrophenol (Group 2) had a similar pattern of hypercholesterolemia. The cholesterol-thyroid birds (Group 3) had consistently lower plasma cholesterol levels throughout the first ten weeks of the experiment. They had a value of about 300 mg. per cent (control: approximately 100 mg. per cent), less than one-half that of the other two



	% WITH LESIONS	AVE. GROSS GRADING	% WITH GRADING 1 OR >
1.	87	2.5	81
2.	74	2.25	58
3.	61	1	26

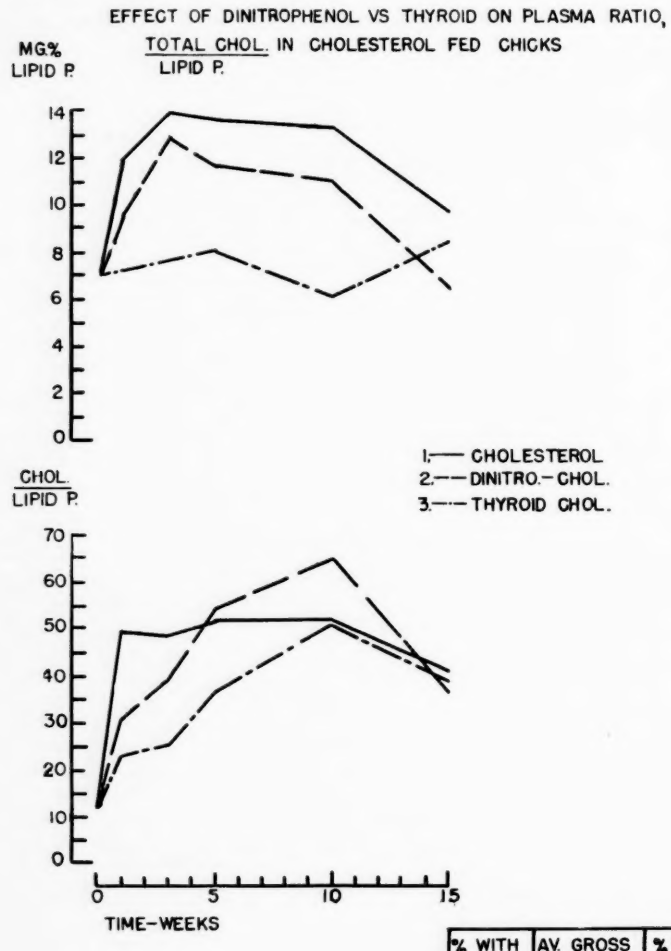
Fig. 1.—Plasma cholesterol levels (graph) and gross pathologic gradings of atheroma (table, lower right) in chicks fed cholesterol (Group 1), dinitrophenol plus cholesterol (Group 2), and thyroid plus cholesterol (Group 3). The control birds fed plain mash (Group 4) showed a uniform plasma cholesterol level of about 100 mg. per cent (see level of three experimental groups at onset of experiment—0 weeks) throughout the experiment. These control chicks were free of gross atheromatous lesions at the end of the experiment (see Fig. 3). Discussed in text.

cholesterol-fed groups. At the end of fifteen weeks all three experimental groups had a similar degree of hypercholesterolemia, due to a fall in plasma cholesterol levels in Groups 1 and 2.[21, 22] Throughout the experiment, no groups showed a significant change in the ratio, $\frac{\text{plasma free cholesterol}}{\text{plasma total cholesterol}}$.

All the cholesterol-fed groups except Group 3 (thyroid plus cholesterol) exhibited a hyperphospholipidemia (Fig. 2). At no time was the hyperphospholipidemia as marked as the hypercholesterolemia. Hence all three experimental groups had an elevated ratio, $\frac{\text{plasma total cholesterol}}{\text{plasma lipid phosphorus}}$. This elevation was considerably less marked in the birds fed thyroid plus cholesterol (Group 3, Fig. 2). During the latter half of the experiment the three groups had similar ratios.

The data on aorta and liver lipids at the end of the experiment are presented in Table II and Fig. 3. All three cholesterol-fed groups had elevated aorta

cholesterol values. The increment was somewhat less in the thyroid-cholesterol group than in the other two experimental groups. No significant changes in aorta phospholipid were noted.



	% WITH LESIONS	AV. GROSS GRADING	% WITH GRADING 1 OR >
1.	87	2.5	81
2.	74	2.25	58
3.	61	1	26

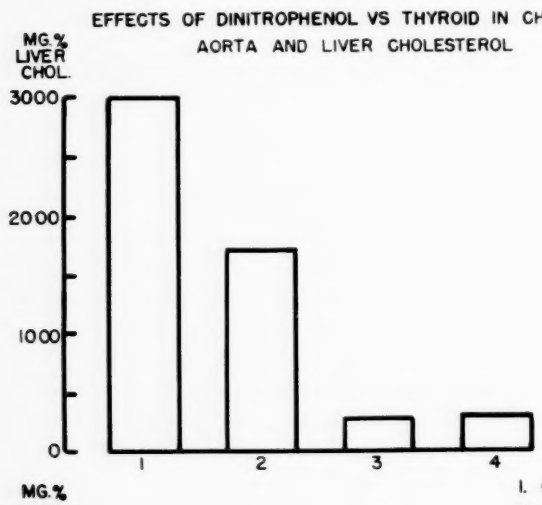
Fig. 2.—Plasma lipid phosphorus levels (*Lipid P*, upper graph), ratios $\frac{\text{plasma cholesterol}}{\text{plasma lipid phosphorus}}$ ($\frac{chol.}{Lipid\ P}$, lower graph), and gross pathologic grading of atheroma (table, lower right) in chicks fed cholesterol (Group 1), dinitrophenol plus cholesterol (Group 2), and thyroid plus cholesterol (Group 3). The plasma lipid values at 0 weeks are controls. The control chicks fed plain mash (Group 4) maintained approximately these levels throughout the experiment. These control birds were free of atheroma at postmortem (see Fig. 3). Discussed in text.

The liver lipid pattern was markedly different among the three experimental groups (Table II and Fig. 3). Groups 1 and 2 had greatly elevated hepatic cholesterol levels; phospholopid was moderately increased; total fatty acid was

TABLE II. AORTA AND LIVER LIPIDS AFTER 16 TO 17 WEEKS ON THE VARIOUS EXPERIMENTAL DIETS LISTED IN TABLE I

	GROUP	LIPID P (MG. %)	PHOSPHOLIPID* (MG. %)	PHOSPHOLIPID FATTY ACID†	TOTAL CHOL.‡ (MG. %)	FREE CHOL. (MG. %)	ESTERIFIED CHOL. (MG. %)	RATIO F/T CHOL.§	RATIO: T. CHOL. / LIPID P	ESTERIFIED CHOL. FATTY ACID‖ (MG. %)	TOTAL FATTY ACID (MG. %)	NEUTRAL FAT FATTY ACID¶	TOTAL LIPID (CALC.)** (MG. %)
Liver	1	120	3123	2092	2995	1133	1862	.38	24.5	1359	4870	1419	8896
	2	106	2783	1865	1710	534	1176	.31	16.1	859	3950	1226	6578
	3	78	2040	1367	278	178	100	.64	3.6	73	4540	3100	5491
	4	86	2230	1494	287	277	10	.95	3.3	7	5340	3839	6363
Aorta	1	33.6	876	587	354	234	120	.65	10.7	88	----	----	----
	2	30.2	779	522	330	231	99	.70	10.9	72	----	----	----
	3	37.1	963	645	271	207	64	.76	7.3	47	----	----	----
	4	33.8	878	588	174	168	6	.97	5.1	4	----	----	----

*Phospholipid: lipid P × 26.
†Phospholipid Fatty Acid: phospholipid × .67.
‡Chol.: cholesterol.
§Ratio F/T Chol.: ratio of free to total cholesterol.
‖Esterified Chol. Fatty Acid: esterified cholesterol × .73.
¶Neutral Fat Fatty Acid: total fatty acid—(phospholipid fatty acid plus esterified cholesterol fatty acid).
**Total Lipid (Calc.): phospholipid plus total cholesterol plus esterified cholesterol fatty acid plus neutral fat fatty acid.



	% WITH LESIONS	AVE. GROSS GRADING	% WITH GRADING 1 OR >
1.	87	2.5	81
2.	74	2.25	58
3.	61	1	26
4.	0	0	0

Fig. 3.—Liver (upper graph) and aorta (lower graph) total cholesterol levels, and gross pathologic grading of atheroma (table, lower right), in chicks fed cholesterol (Group 1), dinitrophenol plus cholesterol (Group 2), thyroid plus cholesterol (Group 3), or plain mash (Group 4). Discussed in text.

essentially unchanged; neutral fat fatty acid was reduced; total lipid was normal or slightly elevated. These alterations from control findings were somewhat more marked in Group 1 (cholesterol mash) than in Group 2 (dinitrophenol-cholesterol mash). In contrast, the Group 3 birds (thyroid-cholesterol mash) had normal liver total cholesterol, phospholipid, and total fatty acid. Except for a decreased ratio, $\frac{\text{free cholesterol}}{\text{total cholesterol}}$, the hepatic lipid pattern of these cholesterol-fed birds had been restored to normal by addition of thyroid to the diet.[23]

TABLE III. GROSS PATHOLOGIC GRADINGS OF AORTAE OF INDIVIDUAL CHICKS IN VARIOUS EXPERIMENTAL GROUPS LISTED IN TABLE I*

	GROUP 1					GROUP 2					GROUP 3				
	BIRD NO.	WEEKS ON DIET	GRADING THOR.	ABD.	SUM	BIRD NO.	WEEKS ON DIET	GRADING THOR.	ABD.	SUM	BIRD NO.	WEEKS ON DIET	GRADING THOR.	ABD.	SUM
Subgroup:			*A1*					*A2*					*A3*		
	577	10	1½	3	4½	178	12	¾	½	1¼	565	16	¼	1	1¼
	401	10	0	0	0	169	12	3	0	3	189	16	¼	0	¼
	485	10	2½	¼	2¾	166	15	1	2	3	188	16	2½	½	3
	410	16	2½	1	3½	170	15	3	0	0	192	16	¼	½	¾
	437	16	3½	3	6½	536	16	3½	0	0	184	17	0	0	0
	544	17	½	1	1½	168	16	3	1½	4½	190	17	0	0	0
	531	17	¼	0	¼	173	16	¼	0	¼	187	17	2½	½	3
	330	17	4	0	4	174	16	3	0	3	183	17	½	½	1
						550	16	2½	0	2½	182	17	1½	1½	3
						359	16	3	0	3	181	17	0	½	½
						569	16	0	0	0					
Mean		–	1¾	1	2¾		–	3	½	3½		–	1	½	1½
Subgroup:			*B1*					*B2*					*B3*		
	477	15	1½	2	3½	198	15	0	0	0	219	16	¼	0	¼
	20	15	2	0	2	201	16	½	1½	2	241	16	0	½	½
	156	15	1	½	1½	680	16	½	1	1½	364	16	0	0	0
	627	15	¼	1	1¼	204	16	¼	0	¼	218	16	0	½	½
	629	15	3½	0	3½	373	16	4	¼	4¼	217	17	0	1	1
	692	15	0	0	0	207	17	0	0	0	226	17	0	0	0
	625	15	1½	0	1½	529	17	½	0	½	–	17	0	0	0
	622	15	1¾	0	1¾	631	17	3	0	3	237	17	0	0	0
											238	17	0	0	0
											–	17	0	0	0
											–	17	0	¼	¼
											–	17	0	¼	¼
											223	17	0	0	0
Mean		–	1½	½	2		–	1½	½	2		–	0-¼	½	½

Thor., Thoracic aorta and brachiocephalic vessels.

Abd., Adbominal aorta and iliac vessels.

*The chicks of the control group, Group 4, were all free of gross atheroma at the conclusion of the experiment; hence their individual gradings are not included in this table.

Vascular Gross Pathology.—Complete gross pathologic data on birds surviving beyond the ninth experimental week are presented in Table III. These findings are summarized in Figs. 1, 2, and 3. The control birds (Group 4) were free of gross lesions at sacrifice at fifteen to seventeen weeks. The chicks fed plain cholesterol mash (Group 1) and dinitrophenol-cholesterol mash (Group 2) had a similar incidence, extent, and severity of atherosclerosis. By contrast, the thyroid-cholesterol birds (Group 3) had significantly less extensive and severe atherosclerosis than the other two cholesterol-fed groups. However, unlike the controls (Group 4) these thyroid-cholesterol fed birds were not completely free of atherosclerosis (Fig. 3).[1, 2] The birds fed thyroid plus cholesterol plus cottonseed oil tended to have a decreased incidence and severity of atheroma compared with chicks fed only thyroid plus cholesterol (Table II). No other significant differences were noted between the paired subgroups subsisting on similar diets except for the presence or absence of 5 per cent cottonseed oil (Table III).

DISCUSSION

The two hypermetabolism-inducing agents, desiccated thyroid and dinitrophenol, differ considerably in their effects upon lipid metabolism and atherogenesis. Unlike thyroid hormone, the pharmacologic agent dinitrophenol has little effect on plasma lipids in cholesterol-fed chicks. This finding is in accord with results reported in a similar study on man.[24] Further, our data show that dinitrophenol, unlike thyroid, does not exert a lipotropic action against cholesterol fatty livers.[23] It has little or no effect on aorta cholesterol concentration in cholesterol-fed chicks. It is ineffective against cholesterol-induced atherosclerosis, whereas desiccated thyroid significantly lowers aorta cholesterol levels and decreases the incidence, extent, and severity of atheroma.

Presumably these different effects are not attributable to a less marked hypermetabolism in the dinitrophenol-fed birds, since the drug dosage was progressively increased to toxic levels and maintained at a concentration slightly below the upper limit of tolerance.[15] The dosage of desiccated thyroid was also below the toxic level.[1]

Since desiccated thyroid significantly alters lipid metabolism and atherogenesis in the cholesterol-fed chick, whereas dinitrophenol does not, these effects of thyroid hormone apparently cannot be attributed solely to the generalized increase in energy exchange it induces. Rather than being nonspecific by-products of increased metabolic rate, these actions of thyroid on cholesterol metabolism would appear to be effected via specific metabolic reactions involving hormone and lipid. Data from other studies on man and animals support this conclusion.[9, 16, 24-28] The specific pathways mediating these effects are obscure, since little is known concerning the intermediary biochemical reactions linking thyroid hormone and the lipids.

The present study extends our findings on the changes in lipid metabolism brought about by feeding thyroid. It confirms previous data showing that this hormone partially inhibits dietary hypercholesterolemia and athero-

sclerosis.[1] It further demonstrates an inhibition of the accompanying generalized hyperlipemia and hyperphospholipidemia, with a reduction of the ratio $\frac{\text{plasma total cholesterol}}{\text{plasma lipid phosphorus}}$. Further, this hormone is shown to be partially effective against aortic cholesterosis. As previously noted, it is almost completely effective as a lipotrophic agent against the cholesterol-type fatty liver.[23]

Recent studies have emphasized three possible factors that may be of significance in the pathogenesis of experimental cholesterol-induced atherosclerosis: (1) hypercholesterolemia, (2) hepatic cholesterosis, (3) elevation of the ratio $\frac{\text{plasma total cholesterol}}{\text{plasma lipid cholesterol}}$.[8, 10-12, 29, 30] The exhibition of desiccated thyroid to cholesterol-fed chicks tends to correct each of these biochemical abnormalities. Except for an increase in hepatic esterified cholesterol and a resultant decrease in the ratio of free to total cholesterol in the liver, the hormone is completely effective in preserving a normal hepatic lipid pattern in the cholesterol-fed chick.[23] The concentration of liver phospholipid, fatty acids, and total cholesterol is normal. Nevertheless, hyperlipemia and atherosclerosis are not completely prevented; the antiatherogenic action of thyroid is incomplete.* From these findings, it may be concluded that cholesterol-induced hyperlipemia and atherosclerosis may develop in the chick in the presence of little or no hepatic cholesterosis. Apparently atherogenesis may proceed without a cholesterol-type fatty liver.

SUMMARY

1. Desiccated thyroid has a partial inhibitory effect on cholesterol-induced hypercholesterolemia and atherosclerosis in the chick. It also lowers the hyperphospholipidemia and hyperlipemia of cholesterol-feeding, decreases aortic cholesterosis, and is effective as a lipotropic agent against the cholesterol-type fatty liver.

2. By contrast, the hypermetabolism-inducing agent dinitrophenol has little or no effect on plasma or tissue lipid levels. It is also ineffective as a prophylactic agent against cholesterol-induced atherosclerosis in the chick.

3. It is concluded that the effects of thyroid on lipid metabolism and atherosclerosis are not mere nonspecific by-products of the induced generalized increase in metabolic rate. Thyroid hormone and the lipids appear to be closely linked metabolically. The hormone apparently influences cholesterol metabolism and atherosclerosis via specific intermediate biochemical reactions in which it participates.

4. Cholesterol-induced atherosclerosis in the chick may develop with little or no increase in the hepatic total cholesterol concentration.

ADDENDUM

Since this paper was submitted, Wolff and co-workers have reported that dinitrophenol-induced hypermetabolism in rats is associated with a reduced plasma protein-bound iodine.[32] In contrast, hyperthyroidism is characterized by an elevated plasma protein-bound io-

*By contrast, Page and Bernhard[31] found that an organic iodide compound protected rabbits against cholesterol-induced atherosclerosis without reducing hypercholesterolemia.

dine.[33, 34] These findings together with our data suggest that protein-bound iodine may be a key regulatory factor in the plasma lipid response to cholesterol feeding. It would appear that iodine and the lipids are intimately related in intermediary metabolism.

We wish to acknowledge our indebtedness to the technical team whose efforts were essential for the execution of this study, and especially to Dr. Ruth Pick, histologist, Miss Marilyn Dudley and Mrs. Eva Levinson, chemical technicians, and Mr. P. Johnson and Mr. G. Crowley.

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DEFECTIVE FAT ABSORPTION FOLLOWING VAGOTOMY

H. J. Fox, M.D.,* and K. S. Grimson, M.D.†
Durham, N. C.
With the Technical Assistance of Anne Jewell, A.B.

IN RECENT years, complete vagotomy has been introduced[1] as a surgical treatment for peptic ulcer. Between June 1941 and June 1948 results in 104 patients treated by this procedure have been determined here and reported.[2] Even though vagotomy with gastroenterestomy has produced encouraging results as a treatment for peptic ulcer, there have occurred certain unexpected complications in a few patients. A small number so treated have presented postoperative complaints of meteorism, episodes of loose stools, or failure to regain normal weight. The number of patients with such complaints constitutes a small percentage of the entire group. Extensive studies here and elsewhere have to our knowledge not included any fat balance tests.

This preliminary report is concerned only with the determination and significance of fat in the feces, and a simultaneous roentgenologic study of the small bowel.

MATERIALS AND METHODS

Two groups of patients comprised the basis for these studies. The first group consisted of nine normal patients convalescent from ailments unrelated to the gastrointestinal tract. In the second group there were nine patients, three of whom had had vagotomy alone and six had had vagotomy with gastroenterostomy as surgical treatment for intractable peptic ulcer. These nine patients were selected from the 104 because of symptoms, such as diarrhea or bowel cramps, indicating dysfunction of the small intestine.

A roentgenologic study of the stomach and small bowel was made on each patient from both groups.

A fat balance test based on the measured intake and output of fat is the only criterion by which normality of fat absorption can be judged.[3] Therefore, both groups were studied in the following manner. A special diet containing 60 Gm. of fat, 100 Gm. of protein, and 300 Gm. of carbohydrate was used. Patients were placed on this diet for a period of at least two days before starting stool collections. Daily measurements of the fat ingested were carefully recorded.

The method for stool collection was similar to that[3] previously described. The collection period was begun after the evening meal by giving an enema of 500 c.c. normal saline and discarding the returns. Thereafter, all stools were collected into a single container for a period of three days. The collection period was ended in the evening, at the same hour as used for starting, after giving another saline enema and adding all returns from this enema to the stool collection.

From the Departments of Medicine and Surgery of Duke University School of Medicine.
Aided by a grant from the Markel Foundation* and a U. S. Public Health Service Grant.†
Received for publication, Oct. 31, 1949.
*Associate in Medicine.
†Professor of Surgery

For estimation of fecal fat, the wet extraction[3] method was used. The feces were measured and weighed; water was added to make a thick creamy mixture; and the specimen was mechanically stirred until homogeneous. Two 20 c.c. aliquot portions were taken and each was placed into special extraction tubes. To one of the samples, 5 c.c. of concentrated HCl were added. The samples were heated on a hot water bath (60° C.) for fifteen minutes, then cooled. To each aliquot portion were added 20 c.c. of ethyl alcohol. The sample tubes were then placed inside large extraction tubes containing ether; the apparatus was connected to condensers, and a hot water bath was used to boil the ether. This continuous ether extraction was carried on until the supernatant layer of ether was colorless even after the addition of a little more alcohol. This usually took three hours or longer. When complete, the ether and alcohol were removed under reduced pressure at 50° C. The dry residue was extracted three times with 10 c.c. of petroleum ether and filtered into weighed flasks. After removal of the solvent, the total fat and fatty acid fractions were weighed. The amount of fat absorbed is equal to the difference between the quantity ingested and the fecal fat.

RESULTS

The results are summarized in Tables I, II, and III.

TABLE I. CHARACTER AND VOLUME OF STOOLS FOR 72-HOUR PERIOD

CASE	SEX AND AGE (YR.)	NUMBER OF STOOLS DAILY	CHARACTER	TOTAL VOLUME (C.C.)
		Normal		
1-9	(6 men, 3 women)	1-2	Formed	450-850 Av. 650
		Vagotomy Alone		
10	M 42	2-3	Soft	600
11	M 39	3-4	Soft	1,500
12	M 46	2-3	Soft	700
		Vagotomy With Gastroenterostomy		
13	M 38	2-4	Soft	900
14	M 70	3-4	Liquid	1,300
15	M 39	3-5	Liquid	1,700
16	M 42	1-2	Soft	1,200
17	M 45	2-3	Soft	700
18	M 56	1-2	Soft	1,100

TABLE II. RADIOLOGIC FINDINGS IN SMALL INTESTINE IN PATIENTS FOLLOWING VAGOTOMY

CASE	APPEARANCE	PERCENTAGE DIETARY FAT ABSORPTION	MONTHS AFTER OPERATION
	Vagotomy Alone		
10	Abnormal, dilated segments	83	18
11	Normal	89	44
12	Normal	93	10
	Vagotomy and Gastroenterostomy		
13	Segmentation, moderate	82	6
14	Segmentation, moderate	77	6
15	Normal	83	19
16	Normal	86	36
17	Segmentation, moderate	82	1
18	Normal	88	22

TABLE III. 72-HOUR FAT BALANCE TEST IN NORMAL PATIENTS AND PATIENTS WITH VAGOTOMY
(DIET: 60 GM. FAT OFFERED DAILY)

CASE	SEX AND AGE (YR.)	DAILY FAT INTAKE (GM.)	DAILY FAT LOSS (GM.)	PERCENTAGE ABSORPTION
		Normal		
1-9	(6 men, 3 women)	46-60 Av. 56	2-4 Av. 3	92-97 Av. 95
		Vagotomy Alone		
10	M 42	60	10	83
11	M 39	57	6	89
12	M 46	60	4	93
		Vagotomy With Gastroenterostomy		
13	M 38	57	10	82
14	M 70	55	12	77
15	M 39	43	7	83
16	M 42	60	8	86
17	M 45	60	11	82
18	M 56	54	6	88

Normal Patients.—The normal patient had an average fecal output for a three-day period of 650 c.c., amounting to 780 Gm. weight.

There were no abnormalities seen in roentgenologic examination of the stomach and small bowel of this group.

A normal human adult on a diet containing 60 Gm. of fat daily absorbed 92 to 97 per cent of the fat ingested. Absorption of 90 per cent or less is regarded as defective. The group of nine normal subjects showed an average absorption of 95 per cent.

Vagotomy Patients.—The fecal output for a three-day period in this group ranged from 600 to 1,700 cubic centimeters. The average for the group during this period was 1,077 c.c., amounting to 1,290 Gm. weight.

The roentgenologic studies on this group as noted in Table II showed that four of the nine patients had localized dilated segments and coarsening of mucosal pattern in the small bowel. In the remaining five out of the nine of this group, no abnormalities were observed during roentgenologic study.

Fat absorption in this group was found to be defective, amounting to less than 90 per cent in all except one patient.

DISCUSSION

The fat balance test in normal adults serves only to demonstrate that normal individuals absorb the dietary fat quite well. This test serves as a quantitative criterion for assessment of normality. Our observations were made on patients stabilized on a diet containing 60 Gm. of fat daily, and under these conditions a fat absorption of less than 90 per cent cannot be regarded as normal. The results obtained in patients experiencing difficulty following vagotomy revealed defective absorption of fat in eight out of nine patients. Also, the average fecal output for this group exceeded in bulk that of the normal patients.

Interpretation of changes in the chemical characteristics of fecal fat is at present unsatisfactory. Although our method included a determination of the percentage hydrolysis of fecal fat, the variations from case to case were consider-

able, and therefore not useful for clinical interpretation. This is in agreement with previous[3] observations. Nor was there any close correlation between the percentage of fat absorption and the roentgenologic pattern of the small bowel.

The logical explanation for the defective fat absorption observed following vagotomy would seem to be that a fault results from interruption of the parasympathetic nerve supply to the bowel, biliary tract, or pancreas. Previous investigators[4, 5] have noted that serious disturbances in gastrointestinal function are frequently found in the diverse manifestations of autonomic nerve disease of diabetic neuropathy. The influence of the parasympathetic nerves on intestinal absorption appears to be of greater importance than that of the sympathetic. Abnormal fat absorption in patients following subtotal gastric resection has been reported.[6]

Although fat balance studies were not obtained on our patients before vagotomy, it is noteworthy that none of them had any of the complaints relating to small bowel dysfunction which subsequently followed vagotomy.

Treatment of this disorder is being investigated. Each patient has been placed on a low fat, high protein, high caloric diet. In addition, two have received folic acid. Although these nine patients were active and fairly well nourished before treatment, early observations show that at least two have responded by gain in weight and strength.

SUMMARY

Fat balance tests were carried out on a group of nine normal patients and nine patients who were experiencing symptoms indicating dysfunction of the small intestine following vagotomy. The normal control patients absorbed more than 90 per cent of the fat ingested. In all of the vagotomy group of patients except one, less than 90 per cent of the ingested fat was absorbed.

The vagotomy group showed an average fecal output which was greater than that of the normal controls.

The roentgenologic pattern of the small bowel was found to be abnormal in four out of nine of the vagotomy group.

These results suggest that the defective fat absorption occurring in certain patients following vagotomy is primarily due to interruption of the parasympathetic nerve supply.

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THE ASSAY AND DISTRIBUTION OF SECRETIN

M. H. F. Friedman, Ph.D., and J. E. Thomas, M.D.
Philadelphia, Pa.

INTRODUCTION

THE purpose of the present study was to develop a procedure for the standardization of secretin and to determine the distribution of secretin throughout the gastrointestinal tract. The need for a suitable assay procedure became apparent while we were trying to prepare a "physiologically pure" secretin for intravenous administration.[1] The methods previously described were found to be either uneconomical in both animals and time or unreliable for reasons to be discussed later.

More exact information on the concentration of secretin in different parts of the small intestine was needed for theoretical as well as obvious practical reasons. In studies such as those dealing with the etiology of fibrocystic disease of the pancreas,[2] the formation of granular complexes in various cellular units of the intestine,[3] the etiology of duodenal ulcer,[4] and the incidence of marginal ulcers following experimental gastroenterostomy,[5] the secretin mechanism has been implicated, but their significance would become clearer only when more exact data on secretin distribution in the gastrointestinal tract become available.

The units of secretin potency used most widely have been the clinical unit of Agren and Lagerlof[6] and the Ivy dog threshold unit.[7, 8] For clinical studies the Swedish investigators established their clinical unit as equal to 16 cat units as defined by them.[9] The Ivy dog threshold unit,[7, 8] at first estimated to be equivalent to 20 Agren cat units, was determined by Greengard and Stein[10] to equal 16 cat units. Thus, by fortunate accident, the Ivy dog threshold unit and the Agren clinical unit are the same and an identical unit was used in this study.

RESULTS

Assay of Secretin Activity.—A potent nontoxic preparation of secretin was prepared for use as a standard as described elsewhere,[1] and the amount necessary to cause an increase in the secretion of 0.4 c.c. of pancreatic juice in ten minutes was determined. The average of the results obtained in eleven dogs was designated the dog threshold (or clinical) unit. This was determined to be .14 mg., with a range of .11 to .17 milligrams. Weighed amounts of this preparation were sealed in ampules and designated as primary standard secretin. To conserve the primary standard, most preliminary assays were made with a secondary standard similarly prepared, and the primary standard was reserved for final evaluations of potency.

Assays were made on dogs (body weight, 10 and 15 kilograms) under sodium pentobarbital anesthesia. The duodenum was drawn out through a laparotomy incision, the accessory duct divided between ligatures, and, with

From the Department of Physiology, Jefferson Medical College.
Received for publication, Nov. 14, 1949.

minimal traumatization, the main duct cannulated at the point on the serosal side where the duct passes into the musculature of the duodenum. By means of a fine rubber tube passing through either a stab wound or the original laparotomy incision the cannula was connected with the recording apparatus. In our studies we used a calibrated Wesson[11] type of drop recorder in which the pancreatic juice displaced distilled water. The abdomen was closed with clamps.

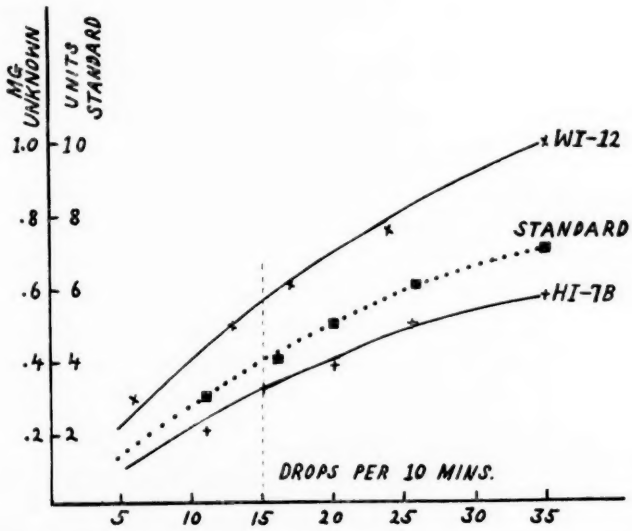

Fig. 1.—Pancreatic secretion in a dog weighing 12.5 kilograms. Dose-response curves for the standard secretin (broken line) and two test concentrates (solid lines) are shown.

All injections were made by femoral vein. A priming dose of a potent preparation of secretin was given to test the patency of the cannula, etc., and to clear the ducts of accumulated pancreatic secretion. The standard secretin and the preparation to be tested were made up to the desired concentration in 1 or 2 c.c. of saline. Immediately following each injection 3 c.c. of physiologic saline were given through the same femoral vein cannula to insure injection of all the secretin.

The standard and the unknown were tested alternately, using a wide range of dosage. The effects of each injection usually lasted less than ten minutes. However, to avoid cumulative effects an interval of twenty minutes between injections was maintained. If the secretory response lasted longer than ten minutes, the interval between two injections was increased to thirty minutes.

The number of drops of pancreatic juice secreted during a ten-minute period immediately following injection was measured. After data had been obtained with a sufficient range of dosage of both standard and unknown, dose-response curves were plotted as in Fig. 1. The abscissas are calibrated in drops per ten minutes, the ordinates in secretin units for the standard and

in milligrams for the unknown. From the intercepts of the chosen ordinate with the curves for the standard and unknown, the potency of the unknown in units per milligrams may be calculated. Thus in the representative dose-response curve shown in Fig. 1 the ordinate corresponding to 15 drops intersects the curves of the standard secretin at 4.0 units, of unknown preparation No. WI-12 at 0.55 mg., and of unknown preparation No. HI-7B at 0.32 milligram. The secretin activity of each preparation was:

$$\text{Concentrate No. WI-12} — \frac{4.0}{0.55} \text{ or } 7.2 \text{ units per mg.}$$

$$\text{Concentrate No. HI-7B} — \frac{4.0}{0.32} \text{ or } 12.5 \text{ units per mg.}$$

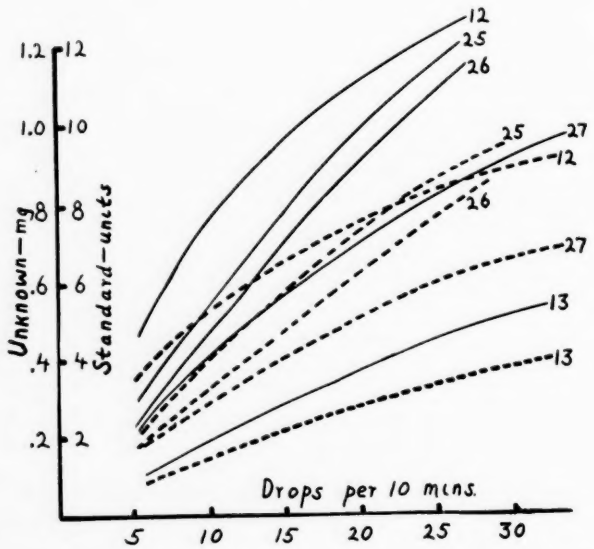

Fig. 2.—Pancreatic secretion in five dogs (Dogs 12, 13, 25, 26, and 27) in response to the same standard secretin (broken line) and the same test concentrate of secretin (solid line).

It will be noted from Fig. 2 that while the pancreatic response to secretin varied widely in different animals, the ratio of response to different secretins was surprisingly constant. Table I shows a typical assay. Two or even three test concentrates could be assayed on each animal providing each injection was made alternately with an injection of the standard secretin. Acceptable assays of two or three preparations were made on as few as five animals.

An interesting observation made in the course of our assay studies was the apparently spontaneous shift in responsiveness to secretin exhibited in certain animals. Our records show this alteration in responsiveness to be nearly always in the direction of increased sensitivity to the secretin. Two explanations suggest themselves: either the pancreas during the operation was subjected to a certain degree of trauma which at first inhibited pancreatic secretion, or else new secretory units, erstwhile dormant, came into activity on repeated excitation with submaximal doses of secretin.[12] Whatever the ex-

TABLE I. PANCREATIC SECRETION IN 6 DOGS IN RESPONSE TO A TEST CONCENTRATE AND A STANDARD SECRETIN; THE WEIGHT OF THE UNKNOWN SECRETIN AND THE AMOUNT OF THE STANDARD SECRETIN REQUIRED TO GIVE A CORRESPONDING RESPONSE AT TWO DIFFERENT LEVELS ARE SHOWN

	PANCREATIC SECRETION IN 10 MIN.					
	15 DROPS			25 DROPS		
DOG	TEST SECRETIN (MG.)	STANDARD SECRETIN (UNITS)	UNITS PER MG.	TEST SECRETIN (MG.)	STANDARD SECRETIN (UNITS)	UNITS PER MG.
12	.96	6.4	6.7	1.23	8.2	6.2
13	.28	2.0	7.1	.45	3.3	7.3
25	.78	5.8	7.4	1.16	8.6	7.4
26	.70	4.8	6.8	1.09	7.6	7.0
27	.57	4.0	7.0	.82	5.9	7.2
31	.62	4.5	7.2	.91	6.0	6.6

planation, these observations demonstrate the unreliability of assay methods based on simple measurement of secretory response alone and emphasize the need for repeated testing with the standard secretin. Since the latter method must, at present, be relied on to determine the potency of any standard, it is evident that secretin assays from different laboratories will never be strictly comparable until a common standard is available.

Distribution of Secretin.—Studies on the distribution of secretin were made in dogs. The intestine was removed immediately after death and divided into segments. Corresponding segments from several animals were pooled and secretin was extracted as described by us.[1] For purposes of comparison the pyloric mucosa of the dog's stomach was similarly treated and assayed for secretin activity. All segments were extracted twice to insure maximum recovery of the secretin. To avoid losses in activity incident to the extraction procedure, all studies were made on the first acetone precipitate of the methanol filtrate (for details see Friedman and Thomas[1]). This precipitate has been found consistently to be free from vasodilator substances.

In confirmation of other workers we found that the secretin content of the intestinal mucosa decreased progressively with the distance from the pyloric sphincter. The secretin content of the terminal 10 cm. of the ileum was determined to be only about 2 per cent of that found in the first 10 cm. of duodenum. Little or no secretin activity was present in extracts of the mucosa of the gastric antrum. The secretin content found in the upper 60 cm. of the small intestine in fourteen dogs is summarized in Table II. The data show that while the weight of the extract from the more distal portions of the intestine was greater,

TABLE II. AVERAGE SECRETIN CONTENT OF THE UPPER SMALL INTESTINE IN 14 DOGS

DISTANCE FROM PYLORUS (CM.)	WEIGHT OF INTESTINE (GM./CM. INTESTINE)	WEIGHT OF EXTRACT* (MG./GM. INTESTINE)	SECRETIN CONTENT		
			UNITS/MG. EXTRACT*	UNITS/GM. INTESTINE	UNITS/CM. INTESTINE
0-10	1.7	2.07	0.45	0.93	1.61
15-25	1.5	2.53	0.35	0.88	1.47
50-60	1.3	2.90	0.15	0.43	0.57

*To avoid loss in purification the first acetone precipitate was used. It was relatively free from vasodilator substances. For procedure see Friedman and Thomas.[1]

the secretin activity was less. The interesting point is brought out that because of the lesser weight of the intestine per unit length at lower levels, the differences in secretin content per unit weight of tissue is less than the differences per unit length.

DISCUSSION

The usual method of determining the activity of secretin concentrates has been to measure the increase in the rate of flow of pancreatic juice following the injection of the concentrate. In this procedure secretin unitage is expressed either in terms of the amount required to give a standard increase in flow rate[7] or in terms of the ratio of secretory rates due to the test concentrate and a given amount of a standard secretin preparation.[8, 13, 14] These methods all gave considerable variability in results when tested on different animals, and the method of Greengard and Ivy,[8] for example, required at least ten dogs for an acceptable assay.

Wilander and Agren[9] used the number of milliliters of 0.1M acid required to neutralize cats' pancreatic juice flowing into an exposed segment of duodenum as equivalent to the number of cat units of the secretin. This method assumes that only pancreatic juice, and not alkaline duodenal juice as well, was being titrated and is based on the unproved assumption that the bicarbonate output rather than the fluid volume is the true measure of pancreatic secretory activity.

Since completion of this study, three pertinent papers on the subject of secretin assay have been published. Doubilet,[15] using the assay method of Greengard and Ivy,[8] called attention to the role which secretinase may play in invalidating the results by enzymatic inactivation of the secretin and made the interesting suggestion that secretinase inhibitors, such as vitamin K, might be used to stabilize the pancreatic response to secretin. Burn and Holton[16] used a simple acetone-dried powder prepared from duodenal mucosa as a secretin standard. They call attention to the urgent need for an international standard preparation of secretin and suggest the provisional use of the acetone-dried duodenal mucosa for the purpose.

Gershbein, Wang, and Ivy[17] have recently described a method of assay similar in principle to that described in this communication. They use as a standard an "S1" preparation of their secretin of which the "threshold" or unit dose is stated to be 0.25 milligram. Instead of determining equivalent doses of standard and unknown from dose-response curves as in our method, they use a trial-and-error procedure. The response to a given dose of the standard is first determined and "the test concentrate is then administered in an amount yielding the same drop count within one or two drops over the same time interval." Although it is obvious that equivalence of dosage cannot be determined in this way as precisely as from dose-response curves, the errors are probably insignificant compared with the probable error in estimating the potency of any standard.

The findings on distribution of secretin are in general agreement with those of several other investigators[16, 18-21] although Mellanby[20] obtained no evidence for secretin below the level of the upper jejunum in cats. In this connection it is important to bear in mind that species differences have been reported; thus, while secretin is limited to the small intestine of the dog and cat, it is said to be present in appreciable amounts in the large intestine of ruminants.[22]

It has been shown that the threshold to various pancreatic stimuli may be lowered by substances, such as peptones and nitrites, which cause vasodilatation and presumably increase the blood flow to the pancreas.[23, 24] Studies on the comparative effectiveness of various secretin concentrates, therefore, are valid only if they are free of vasodepressor effects, as were those used in this study.

SUMMARY

1. A method for the assay of secretin activity on the anesthetized dog is described using dose-response curves for comparing the activity of the unknown with a standard preparation.

2. Secretin was found present throughout the whole length of the small intestine in the dog, with the greater concentrations occurring within the first 25 cm. from the pylorus. The differences in secretin content per unit weight of tissue from different regions of the intestine were found to be less than the differences per unit length.

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CHYLOMICRONEMIA, FAT TOLERANCE, AND ATHEROSCLEROSIS

JOHN R. MORETON, M.D.
SALT LAKE CITY, UTAH

INTRODUCTION

THE view that the blood may be the immediate source of the depositing lipids in atherosclerosis was suggested by Virchow nearly a hundred years ago. This concept of direct lipid infiltration was supported by Aschoff,[1] Anitschkow,[2] and a number of subsequent authorities. An unanswered question has been whether the primary cause of this deposition of infiltrating lipids resides in abnormality of the arterial wall or of the lipids in the plasma, or both. The findings of many investigators now indicate that marked and sustained elevation of plasma lipid levels is the abnormality common to the known causative conditions of early and severe atherosclerosis in certain syndromes in man and as produced experimentally in animals. In laboratory animals Hueper[3] found that repeated intravenous injections of macromolecular substances such as polyvinyl alcohol, methyl cellulose, and pectin, resulted in their deposition in the arterial wall in lesions morphologically resembling atherosclerosis. (He concluded that the cause of this deposition was dependent on anoxemia, a hypothesis which he has developed in detail.[3, 4]) The deposition in the arterial intima of lipids in hyperlipemia, and of experimentally injected colloidal substances such as polyvinyl alcohol, lend support to the view that the primary cause of intimal deposition is dependent not upon antecedent pathology in the arterial wall but on abnormality in the chemical or physical nature of the substances in the plasma.

Observations on the comparative particle size of the lipids in normal and abnormal serum, and an etiologic mechanism based thereon, were presented in recent papers from this laboratory.[5, 6] Serum from patients and experimental animals with sustained, pathologic hyperlipemia (hypercholesterolemia) was found to contain lipid in the form of coarsely suspended, visible particles, in contrast to the highly dispersed, ultramicroscopic colloidal increments characteristic of normal, fasting serum. Plasma-derived nutrient lymph, which normally infiltrates the arterial wall from the high-pressure column of blood in the lumen, is believed to carry some of these large particles into the intima where they are phagocytosed by macrophages, giving rise to foam cells, the characteristic lesion of active atheromagenesis. In normal persons with normal plasma lipid levels the absorption of a fatty meal results in a temporary flooding of the plasma with myriads of similarly large lipid particles (chylomicrons), thus duplicating the coarse lipid disperson found in sustained hyperlipemia. The theory was advanced[5] that the gradual development of atherosclerosis in otherwise normal human beings may be the direct result of recurrent alimentary chylomicronemia due to the repeated ingestion of many

From the Joseph Edgar Tyree Memorial Laboratory for Research on Arteriosclerosis.
Received for publication, Nov. 17, 1949.

fatty meals over a lifetime. In this paper are presented data demonstrating that considerable differences exist in the severity and duration of chylomicronemia in normal persons following a standard fat test meal.

Fundamental contributions on the physiology of fat digestion and absorption were presented by Gage and Fish[7] and Frazer and Stewart.[8, 9, 10] In dark-field observations on serum these investigators reported the striking appearance of great numbers of chylomicrons following the ingestion and absorption of fat. From serial chylomicron counts in the hours immediately following a fatty meal they plotted curves illustrating the height and duration of this manifestation of fat absorption. In our laboratory, interest in this dark-field method arose out of our hypothesis of etiologic relationship between chylomicronemia and atherosclerosis.

METHODS

Dark-field Counts.—Dark-field counts were made on serum samples from a number of subjects following a standard fat test meal, as well as on sera from patients and experimental animals with sustained pathologic hyperlipemia. For the purpose of reliable comparisons, this technique, at least in our hands, was found to have a number of shortcomings: Microscopic counts enumerate the particles which are large enough to be visible in the dark field but do not reflect the size as well as the numbers of particles (hyper- and macrochylomicronemia). Variations in the intensity of the light source and focusing of the dark-field condenser cause variations in the numbers and sizes of particles made visible by their scattering of incident light. The process of counting is constantly harassed by the Brownian motion of the chylomicrons which causes them to dance in and out of the plane of focus, and in and out of the lateral limits of the field being counted. It is difficult to be certain that the thickness of the film being counted is the same from one preparation to the next (even when using a quartz counting chamber). Counts of a given specimen by different observers were often at rather wide variance and were not closely repeatable by a single observer. Photomicrographs of dark-field preparations using photoflash bulbs as a brilliant source of instantaneous illumination were found to be successful and of interest but still subject to many of the same shortcomings and therefore were not adopted as a routine procedure. When moderate to large amounts of fat were fed (e.g. 50 to 100 grams), the serum of most normal subjects in the postprandial state was teeming with such a thick concentration of visible chylomicrons that counting them, even in a thin film, was virtually impossible. In pathologic, sustained hyperlipemia of metabolic origin the serum is often very turbid, or frankly creamy, and attempts at counting the vast numbers of visible particles in the dark field were quite in vain. From the standpoint of possible clinical adoption, this time-consuming, tedious, and inaccurate technique seemed definitely inadequate. It was evident that another method was needed for the quantitative comparison of chylomicronemia in different serum samples.

Tyndall Effect and Nephelometry.—Lipid particles in dark-field preparations are visible only by their scattering of incident light. It is readily seen that larger lipid particles scatter proportionately more light than smaller ones. Thus, the light-scattering by a solution containing suspended particles increases with the particle size as well as the number of particles present. True solutions, those without suspended particles of colloidal range or larger, appear optically empty. It was apparent that quantitative determinations of the Tyndall light scattered by different serum samples would provide a relatively simple method for comparison of the degree of hyper- and macrochylomicronemia. Using a beam of incident light of constant intensity, those serum specimens that contain the greater number of larger lipid particles scatter the greater amount of light. (The relatively small amount of light scattered by the plasma proteins may be considered part of the normal base line.) From these considerations we constructed an especially designed photoelectric nephelometer for the quantitative measurement of the Tyndall light from normal and chylomicronemic sera.

The Nephelometer: The serum sample is pipetted into a square specimen cell of optical glass. This cell is placed in a precision-built, square specimen housing. The latter is provided with two windows, one for the entrance of incident light and the other, on an adjacent side, for the exit of Tyndall light scattered at right angles to the incident beam. The incident light is provided from a 150 watt projection type of incandescent lamp which is fixed at about 5 inches from the specimen and is made parallel by an interposed lens system. A photoelectric cell of high sensitivity is placed next to the Tyndall window to receive light scattered by suspended particles in the specimen. The instrument must be so constructed

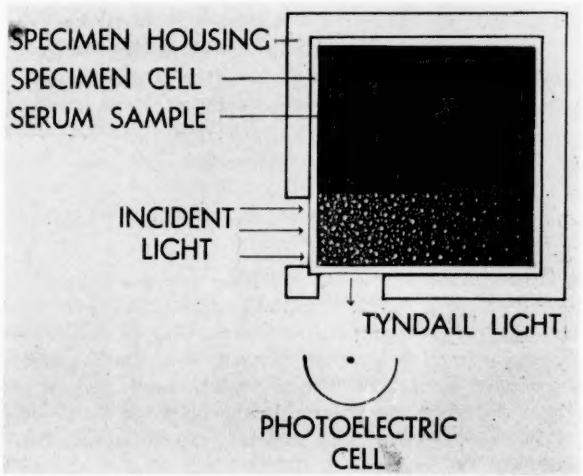

Fig. 1.—Cross section through specimen housing of nephelometer illustrating optimum position of windows. The specimen shown represents a chylomicronemic serum sample taken from a normal subject following the ingestion of a fat-rich meal. The intensity of the Tyndall light is measured in microamperes by the photoelectric cell circuit.

and so shielded that only Tyndall light falls on the photoelectric cell. The electronic circuit, which measures the Tyndall light in microamperes, and the position of the two windows (Fig. 1) are designed for a wide range of linear response. A constant input voltage is maintained by a precision regulator.

The use of this instrument has the advantage over microscopic counts of giving reliable, constant, and repeatable comparative readings on different samples, and of reflecting increased particle size as well as numbers. The presence of increasing numbers of large lipid particles, chylomicrons, causes corresponding increases in the light-scattering as measured nephelometrically in microamperes. It is obvious, however, that this technique does not provide determinations of mean particle size or particle size frequencies, both of which entail vexatious problems for elaborate physicochemical investigations. The nephelometer is so constructed as to be very sensitive and give a linear calibration over a wide range of turbidity, as determined by readings on serial dilutions of other colloidal solutions and suspensions. Serial aqueous dilutions of wax particles in aqueous suspension (Glo-Coat*) or bentonite (Micron Bentonite†) were found to be satisfactory for this purpose. The simplicity of this nephelometric method is such that the degree of hyper- and macrochylomicronemia in many different samples may be quantitatively compared rapidly and accurately, and related to a standard solution.

Nephelometric Standard Solution.—In order that results with different nephelometers may be comparable, it is essential that a common nephelometric standard be used. Such

*Glo-Coat, S. C. Johnson and Son, Inc., Racine, Wis.

†BC Micron Bentonite, American Colloid Company, 363 West Superior Street, Chicago, Ill.

a standard must meet the criteria of simplicity of preparation, exact repetition, and relative stability. After preparing a number of colloidal solutions and fine suspensoids, the following was found to be most satisfactory as a nephelometric standard.

Using reagent grade (chemically pure) glycerine, Na_2SO_4, and $BaCl_2$, two stock solutions are prepared: .005 molar Na_2SO_4 in glycerine, and .005 molar $BaCl_2$ in glycerine. These salts dissolve quite readily with heat, yielding clear solutions. Equal volumes (e.g. 10 c.c.) of .005 molar Na_2SO_4 and .005 molar $BaCl_2$ are placed in separate test tubes and heated in a water bath (brine) to 100° C. The solutions at this temperature are poured together and mixed. A very finely divided precipitate of $BaSO_4$ rapidly forms. This highly dispersed suspensoid is then allowed to cool. When kept at room temperature this suspensoid shows little evidence of settling in the viscous glycerine and gives the same nephelometric reading for at least one week.

We have arbitrarily assigned the value of 100 units to the Tyndall light scattered by this standard solution. All of our nephelometric results on serum samples are converted into these units.

$$\frac{\text{Microamperes of Tyndall light scattered by specimen}}{\text{Microamperes of Tyndall light scattered by standard}} \times 100 = \text{Units of chylomicronemic turbidity of specimen}$$

Fat Tolerance Test.—This test consists of the quantitative comparison by nephelometry of the chylomicronemia in different subjects in the postprandial hours following the ingestion of a standard fat test meal. Because it is believed, according to this chylomicron theory, that the cause of intimal lipid deposition is directly dependent on the physical state of the lipids in the circulating plasma, and unrelated to plasma lipid concentrations, it has seemed meaningless and unnecessary to determine simultaneously the plasma lipid levels during alimentary hyperlipemia. Early in these investigations it became apparent that there are definite and rather marked variations in fat tolerance in different individuals.[5] In the beginning all subjects were given 50 grams of butter with an otherwise fat-free breakfast. (It should be noted that butter is actually about 80 per cent butterfat and 20 per cent water and other substances.) However, it was later found that cream ordinarily constitutes a more practical and palatable test meal than an equivalent amount of butter; further, the test amount of cream is more easily measured volumetrically than the weighing of an equivalent amount of butter. For these reasons a measured amount of heavy (whipping) cream of known fat content was adopted as the standard fat test meal. The heavy cream provided by urban dairies is usually of known butterfat content and is routinely kept at nearly constant values from day to day. Most of the fat droplets and globules in cream are relatively large and require considerable further emulsification before intestinal absorption can take place. Fat tolerance curves in a given subject following the same amounts of fat given as whipping cream or butter were found to be sufficiently comparable that equivalent amounts of butterfat in either form may be assumed to give a similar type of chylomicronemic reaction in the same subject. (Certain vegetable oils, such as olive oil, cottonseed oil, and corn oil were tried, but these are definitely less pleasant for the subject and may on occasion distort the chylomicronemia curve by nausea which results in delayed peristalsis and digestion and delayed absorption from the intestine.)

In fat tolerance studies on different individuals it soon became apparent that it was not reasonable to give all persons, regardless of size, the same test amount of fat. A 220-pound, 6-foot subject should be able to absorb a given test amount of fat with less reaction in his large blood volume than a slight, 100-pound subject with a considerably lesser blood volume and smaller absorptive area in the intestine. For this reason a fat tolerance test was devised which involves the administration of an amount of fat dependent on the size of the individual being tested. According to Griffin and co-workers[11] the blood volumes of different persons are more closely correlated to their surface areas than to either height or weight. It has seemed reasonable to give an amount of fat dependent on the surface area of the subject as indicated by tables computed from the Du Bois height-weight, surface area formula.[12] We arbitrarily give 60 grams of butterfat (200 c.c. of whipping cream of 30 per cent fat content) to a

standard subject 5 feet 10 inches tall, weighing 150 pounds. The amount of fat given to each person is determined by the ratio of his surface area to that of this arbitrary standard subject. It has been found convenient to prepare a table from which one can read directly this calculated amount of fat to be given each subject according to his height and weight. In this way it has been possible to give different individuals a comparable test amount of butterfat in order to observe quantitatively the differences in the resultant chylomicronemia.

Procedure: The night before the test the subject is required to eat an essentially fat-free supper. The fasting blood specimen is taken in the morning before breakfast. The subject should refrain from drinking water or smoking until this first specimen is taken.* The calculated amount of heavy cream of known fat content is then measured in a graduated cylinder, poured into a suitable container, and given to the subject. This test amount of cream is taken with an otherwise fat-free breakfast, such as fruit or fruit juice, cold or hot cereal, toast, and coffee. This test meal is usually eaten within ten to fifteen minutes. Samples of venous blood are taken at hourly intervals thereafter. (Time begins when the subject begins to eat the test meal.) Smoking and water, but no further food, are permissable during the remainder of the test period.

For our instrument each serum sample must be 1.5 to 2 cubic centimeters. Approximately 5 c.c. of blood are withdrawn by venipuncture and allowed to stand in a test tube at room temperature for three to four hours for clot retraction. This tube is then centrifuged at approximately 2,800 to 3,000 r.p.m. (about 600 × gravity) for twenty minutes and the serum pipetted into another tube which is again similarly centrifuged. The serum is then pipetted into a nephelometer cell. In this way the serum is rendered entirely free of all blood cells, fibrin, and platelet fragments. It is essential that hemolysis be scrupulously avoided in the preparation of the serum sample, as red-tinged serum (or icteric serum) absorbs some of the Tyndall light and therefore will cause a falsely low reading in the photoelectric nephelometer. The syringe and test tubes should be clean and dry and essentially free of dust particles.

RESULTS

In the fasting state the serum of normal subjects is clearly transparent and scatters a relatively small amount of Tyndall light (Table I). The highly dispersed plasma proteins and lipids intrinsic in normal serum are responsible for this light-scattering in the fasting state (Fig. 2). In contrast, the large lipid particles, chylomicrons, in alimentary hyperlipemia or sustained pathologic hyperlipemia of metabolic origin are easily visible in the dark-field microscope, cause gross turbidity of the serum, and scatter relatively large amounts of Tyndall light.

Fasting serum samples of all normal subjects scatter about the same amount of Tyndall light. The variations that do exist are not dependent on or related to normal, fasting cholesterol levels (Table I). Following the fat test meal, chylomicronemia usually becomes evident in about thirty minutes and in most persons reaches a maximum between two and four hours, and usually returns to fasting values within six to eight hours. As may have been expected, the height and duration of the chylomicronemia in a given individual was found to be dependent on the amount of fat ingested (Fig. 3). Larger amounts of fat produce a proportionately greater chylomicronemia and one of longer duration, whereas smaller amounts produce a proportionately lesser

*Drinking water or smoking may wash out or squeeze out, by the first active peristalsis of the morning, a few chylomicrons which may in an occasional individual remain in the lacteals as a result of fat ingested the day before. This would result in the erroneous impression of fasting chylomicronemia of internal, metabolic (pathologic) origin.

TABLE I. CHOLESTEROL LEVELS AND PHYSICAL STATE OF LIPIDS IN FASTING SERUM

SUBJECT	CHOLESTEROL LEVEL	NEPHELOMETRIC UNITS	GROSS APPEARANCE	LIPID PARTICLES SEPARATED TO THE TOP BY HIGH-SPEED CENTRIFUGATION (20,000 × G)
Normal adult, fasting	185	92	Clear	0
Normal adult, fasting	214	85	Clear	0
Normal adult, fasting	193	78	Clear	0
Normal adult, fasting	310	84	Clear	0
Normal adult, fasting	242	88	Clear	0
Normal adult, fasting	227	72	Clear	0
Normal adult, fasting	165	76	Clear	0
Normal adult, fasting	213	104	Clear	0
Normal adult, fasting	174	97	Clear	0
Normal adult, fasting	189	81	Clear	0
Normal adult, fasting*	180	216	Slightly turbid	+
Normal adult, fasting	197	79	Clear	0
Normal adult, fasting	210	88	Clear	0
Diabetic subject, with sustained metabolic hyperlipemia, fasting	724	2,725	Markedly turbid	+++
Diabetic subject, with sustained metabolic hyperlipemia, fasting	404	2,240	Markedly turbid	+++
Nephrotic subject, with sustained metabolic hyperlipemia, fasting	467	2,368	Markedly turbid	+++
Idiopathic sustained metabolic hyperlipemia, fasting	570	3,360	Frankly creamy	++++

Serum samples from normal persons in the fasting state appear to have about the same fine, colloidal, dispersion of intrinsic lipids, despite wide differences in cholesterol levels in the normal range. In contrast, sustained pathologic hyperlipemia is usually characterized by a coarse particulate distribution of the blood lipids, a persistent *metabolic chylomicronemia* (as opposed to the temporary *alimentary chylomicronemia* following the ingestion of a fatty meal by a normal person).

*This healthy normal subject consistently showed a mild metabolic chylomicronemia, despite normal cholesterol and other lipid levels; no evidence could be found of any syndrome usually associated with abnormal lipid metabolism. Such subjects appear to be relatively uncommon.

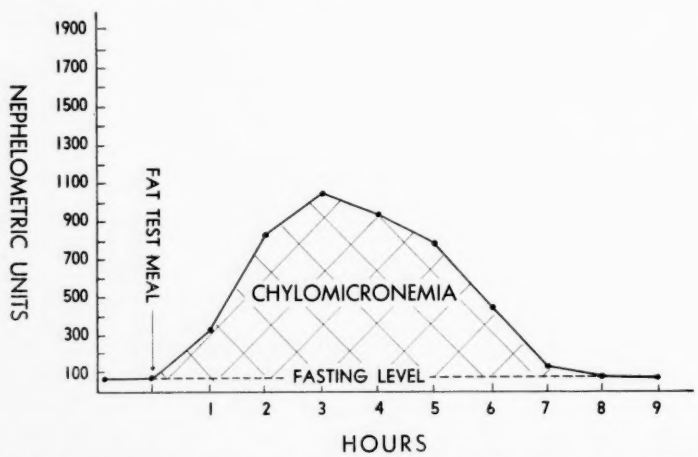

Fig. 2.—Chylomicronemia curve of a normal adult following the fat test meal. The light-scattering in the fasting state was 70 nephelometric units, and at three hours after the fatty meal was 1,045 units. The relatively small amount of light-scattering in the fasting state was due to intrinsic serum lipids in the concentration of 0.732 per cent and proteins in the concentration of 6.40 per cent. At three hours the total lipids in the serum were 1.160 per cent. Thus, the marked increase in light-scattering at three hours was due to chylomicrons which increased the lipid content of the serum by only 0.428 per cent (1.160 minus 0.732).

chylomicronemia, usually of shorter duration. A very small amount of fat (5 to 10 grams), when contained in a large, dinner-size meal of fat-free and essentially fat-free foods, produces virtually no chylomicronemia (Fig. 3).

Considerable variations in fat tolerance were found in different normal subjects as indicated by both the height and duration of their respective chylomicronemia curves (Figs. 4, 5, and 6). The maximum chylomicronemia

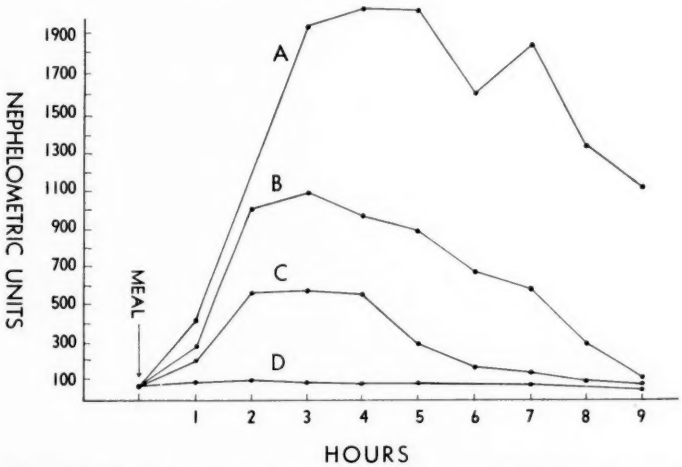

Fig. 3.—Chylomicronemia curves of a normal adult: *A*, following twice the fat content of the fat test meal; *B*, following the fat test meal; *C*, following one half the fat content of the fat test meal; *D*, following an essentially fat-free meal containing lean meat, bread, vegetables, skimmed milk, and fruit.

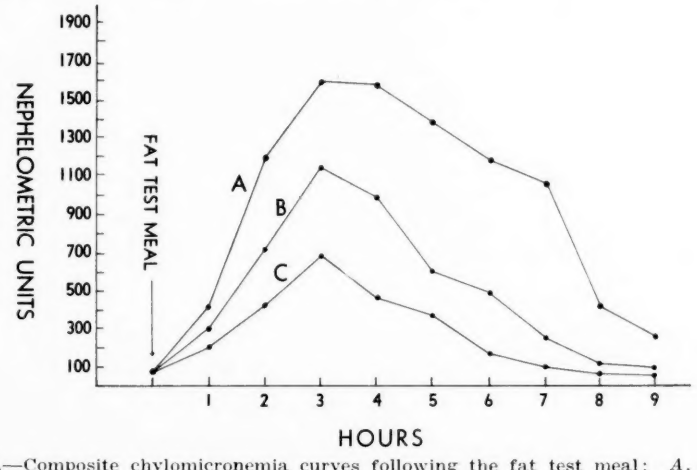

Fig. 4.—Composite chylomicronemia curves following the fat test meal: *A*, of five normal adults showing low fat tolerance; *B*, of ten normal adults showing average fat tolerance; *C*, of five normal adults showing high fat tolerance.

in some subjects with a high chylomicronemia curve was found to be over 100 per cent greater than in subjects with a low chylomicronemia curve. Also, the duration of chylomicronemia was found to be much more prolonged in some persons than in others. Most subjects were found to have chylomicronemia curves within the range shown in Fig. 4. Of particular interest was the finding that repeated tests on the same subjects revealed that fat tolerance tends to remain about the same in each individual (Fig. 5).

A detailed fat tolerance test requires a fasting blood specimen and hourly specimens for six to eight (or more) hours following the standard fat test meal. However, it appears that a fairly satisfactory indication of fat tolerance may be obtained by serum samples taken fasting and at three and six hours. These specimens give an approximate indication of the height and

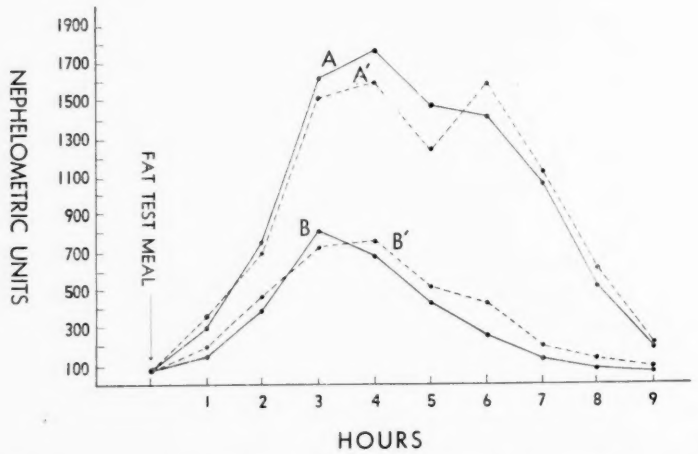

Fig. 5.—Chylomicronemia curves of: tests *A* and *A'*, performed six months apart on a normal adult with low fat tolerance; tests *B* and *B'*, performed six months apart on a normal adult with high fat tolerance.

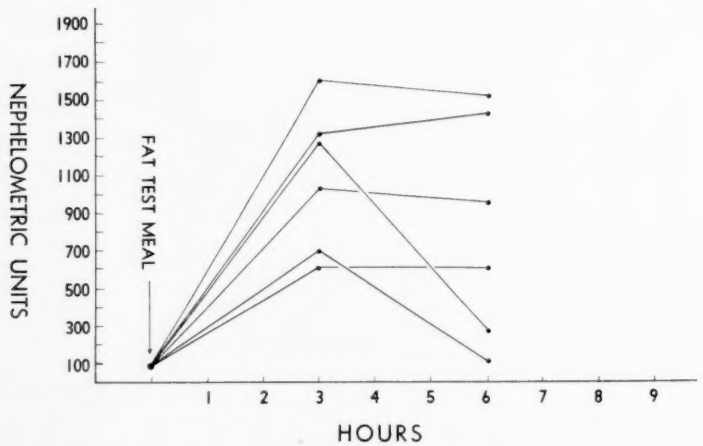

Fig. 6.—Nephelometric readings at three and six hours following the fat test meal in six normal adults. For practical purposes this abbreviated test adequately indicates approximate differences in fat tolerance.

duration of the chylomicronemia (Fig. 6). This simplified estimation of fat tolerance in three venipunctures (instead of eight or nine) would be more expeditious as a practical clinical procedure.

When an individual eats only fat-free and essentially fat-free foods, his serum remains clear and free of large particles throughout the day (Fig. 7). However, when he satisfies a major portion of his daily caloric requirements

by fat in the form of three fat-rich meals, his blood is repeatedly flooded with overlapping periods of hyper- and macrochylomicronemia of alimentary origin (Fig. 7). When a patient with pathologic sustained hyperlipemia eats a fatty meal, the resulting temporary alimentary chylomicronemia is superimposed on the persistent metabolic chylomicronemia (Fig. 8).

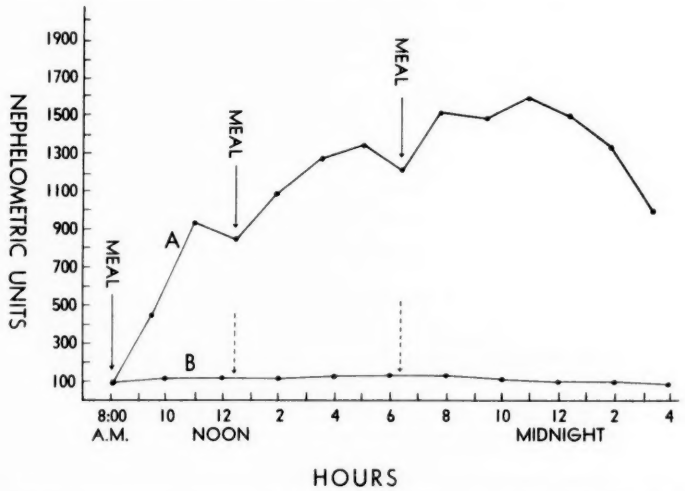

Fig. 7.—Chylomicronemia curves of a normal adult: *A*, following three fat-rich meals, each containing 60 grams of fat; *B*, on a subsequent day, following three essentially fat-free meals (containing the same caloric values as those in *A*).

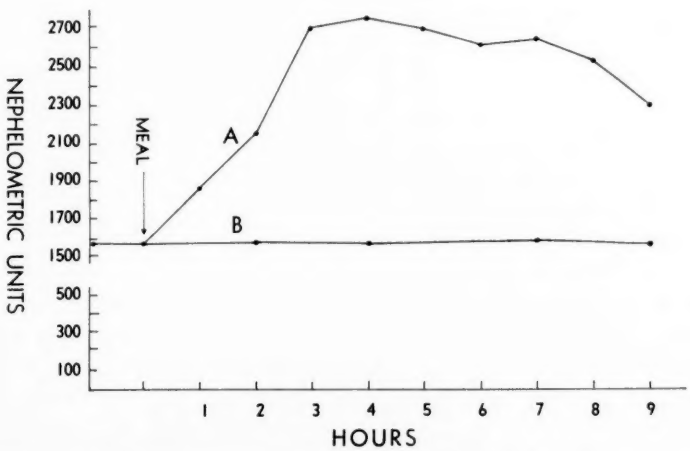

Fig. 8.—Patient with idiopathic sustained hyperlipemia: *A*, chylomicronemia curve after ingestion of the fat test meal; *B*, after an essentially fat-free meal.

DISCUSSION

Normal digestive and absorptive mechanisms are apparently not able to deliver large amounts of newly absorbed fats of alimentary origin to the blood in the same highly dispersed, colloidal state in which the intrinsic lipids and proteins of normal, fasting plasma are carried. The total exposure of the

arterial intima to plasma-derived nutrient lymph containing large lipid particles is a function of both the height and duration of chylomicronemia, and hence is properly represented by the area embraced by the chylomicronemia curve (Fig. 2), rather than by its height or duration alone.

That a person's fat tolerance was found to remain about the same on repeated tests indicates that these differences in normal subjects cannot be attributed to some temporary or changing physiologic or metabolic status. Rather it would seem that an individual's characteristic fat tolerance is in some way a constitutional attribute. It has been found that fat absorption from the intestine is nearly complete (about 95 per cent) in all normal persons.[13-16] Therefore it is doubtful that the minor differences which may exist in the per cent absorption of fat in different individuals are responsible to any significant degree for these observed differences in fat tolerance. In serial roentgen observations following a radiopaque fatty meal, Annegers and Ivy[17] found variations in different persons in the rate of gastric emptying of the fatty meal; these individual differences were similarly manifest on repeated examination. Such differences could be a factor in determining the time of maximum chylomicronemia, but would not explain why some persons regularly exhibit chylomicronemia curves of twice the height and of considerably longer duration than those of others. Frazer,[18] in his interesting studies on the mechanism of fat digestion and absorption from the intestine, reported that when potent preparations of pancreatic lipase were fed with a fatty meal the resulting chylomicrons in the plasma, as seen by dark-field, were greatly reduced in numbers. This finding has been corroborated by the recent observations of Becker, Meyer, and Necheles.[19] This suggests that individual differences in fat tolerance in normal persons may be related to differences in secretion of pancreatic lipase. Other problems for further investigation include the possibility of the relationship of fat tolerance to age, sex, obesity, familial pattern, and other factors. In this regard Becker and co-workers[19] have recently reported that a fat test meal produces a considerably greater and more prolonged increase in chylomicron counts in elderly subjects.

Fat tolerance, as determined by the extent of hyper- and macrochylomicronemia following a standard fat test meal, may be a factor in individual susceptibility to atherosclerosis. Another important variable would seem to be the rate of local infiltration of plasma-derived nutrient lymph which carries large particles, when present in the plasma, into the intima. It is probable that local intimal permeability and intraluminal blood pressure are factors which determine this rate of local infiltration of plasma into the arterial wall. On this basis it may be postulated that some persons, even though on high-fat diets, may, because of high fat tolerance and low intimal permeability, develop very little atherosclerosis. In contrast, other persons on diets containing lesser amounts of fat may, because of low fat tolerance, greater intimal permeability, and high blood pressure, develop severe atherosclerosis and at a relatively young age. It is presumed[5, 6] that neutral fats are readily and rapidly resorbed from deposited chylomicrons, leaving cholesterol as a difficult-to-remove residue. The resistance of cholesterol to resorption is demonstrable

experimentally[20] and is further suggested by its common persistence as an unresorbed residue remaining from infarcts and hemorrhages in many tissues throughout the body. The cholesterol in atherosclerotic lesions may represent slowly accumulated, condensed residues from a great many chylomicrons gradually deposited over prolonged periods.

SUMMARY

The development of atherosclerosis in normal persons with normal plasma lipid levels may be directly attributable to the large particle size of the newly absorbed lipids (chylomicrons) appearing in the plasma following fat-rich meals. The severity of the hyper- and macrochylomicronemia is dependent on the amount of fat ingested. Meals composed of fat-free and essentially fat-free foods do not give rise to chylomicronemia and the serum remains clear and free of large particles.

A fat tolerance test is described in which the amount of fat administered (in the form of heavy cream) is determined by the height and weight of the subject. Serial samples of venous blood are taken at hourly intervals following the fat test meal. The degree of chylomicronemia is estimated by the Tyndall light scattered by the serum sample and is quantitatively measured by photoelectric nephelometry. Nephelometric measurements of Tyndall light are translated into units based on a standard solution. The results of the fat tolerance test are plotted as a curve which shows the height and duration of chylomicronemia following the fat test meal.

Considerable differences in fat tolerance were observed in normal subjects. Repeated tests at intervals of six months showed that an individual's fat tolerance tends to remain rather constant.

Fat tolerance is believed to be a factor influencing individual susceptibility to the gradual development of atherosclerosis from fat-rich meals.

The author wishes to acknowledge with gratitude the advice and assistance of Dr. Louis G. Moench of the University of Utah Medical School and the Salt Lake Clinic.

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THE UTILIZATION OF AMINO ACIDS BY PATIENTS FOLLOWING SURGERY

WILLIAM PARSON, M.D., H. S. MAYERSON, PH.D., ALAN G. C. WHITE, PH.D., AND CHAMP LYONS, M.D.
NEW ORLEANS, LA.

THE nature of the altered nitrogen metabolism following trauma is not known. It has been described as "toxic destruction of tissue" and the "catabolic phase." Subjects who have not been previously depleted usually respond to trauma with a negative nitrogen balance of variable duration which is not necessarily related to the extent of the trauma.[1] It has been claimed by some[2] that this negative nitrogen balance cannot be corrected by increased food ingestion; others[3] have suggested that an abundance of calories can prevent this negative balance.

An attempt has been made to study a phase of nitrogen metabolism during this postoperative period by the use of a "load test." It has recently been demonstrated[4, 5] that rapidly administered amino acid mixtures are well tolerated by patients. Study of the urine reveals a characteristic pattern of amino-nitrogen excretion. These results suggested the possibility of studying the nitrogen metabolism of surgical patients by presenting them with a "load" of amino acid mixture before and after surgery. If the effect of trauma is to interfere with the utilization of amino acids, this procedure might conceivably provide some information as to the nature of the disturbance.

PROCEDURE

Experiments have been performed on four patients. Three healthy subjects served as controls. The studies were done under standard conditions in a metabolic ward. The patients were hospitalized for a period of five to eight days before surgery, placed on full constant diets, and their daily nitrogen balances determined. For these purposes, fecal nitrogen was assumed to be a constant amount, 1.3 Gm. daily. Metabolic studies were not done on the control subjects. As healthy adults who were maintaining constant weight, they were assumed to be in nitrogen equilibrium at the time of the experiments.

The amino acid preparation used was Merck's VUJN preparation.* This is a 10 p.c. mixture of amino acids obtained by complete acid hydrolysis of casein; it contains only minimal amounts of glutamic and aspartic acids and is supplemented with *d,l*-methionine, *d,l*-tryptophane, and glycine. The total nitrogen content is 14.7 Gm. per liter and the α-amino nitrogen content is 12 Gm. per liter.

The tests usually were performed early in the morning. The patients received no food by mouth after midnight of the preceding day. After voiding, an infusion of 500 ml. of normal saline was begun and the rate was adjusted so that the infusion was complete at the end of two hours. The amino acid test infusion was then started and the inflow adjusted so that 500 ml. usually were given in fifty-five minutes. Hourly urine specimens were obtained by voiding for six hours. On a few occasions catheterization was necessary in the postoperative patients.

From the Departments of Medicine, Physiology, Biochemistry, and Surgery, School of Medicine, Tulane University, and the Alton Ochsner Medical Foundation.

This is publication No. 12 from researches conducted under a grant from the Office of the Surgeon General, United States Army.

This study was supported in part by an Institutional Grant from the American Cancer Society.

Received for publication, Nov. 18, 1949.

*The amino acid preparations used in this study were made available to us through the kindness of Dr. Augustus Gibson of Merck & Company, Inc., to whom we are grateful.

Total nitrogen was determined by the micro-Kjeldahl method, urea nitrogen by the urease method, ammonia nitrogen by aeration, and α-amino nitrogen by the ninhydrin method.[6]

A test was performed on each patient on the day before or on the day of surgery and at varying periods thereafter. Patient L. C., aged 44 years, suffered from bronchiectasis and submitted to a lobectomy. Patient J. V., aged 65, had a thyroid carcinoma and a thyroidectomy and tracheotomy were done. Patient B. R., aged 56, underwent a radical mastectomy for breast carcinoma. Patient B. B., aged 18, was admitted to the hospital with a stricture of the esophagus necessitating a jejunostomy, esophagectomy, and esophagogastrostomy. It proved impossible to perform accurate balance studies on this patient.

RESULTS AND DISCUSSION

In general, the infusions were well tolerated. Transient nausea with no vomiting was present in a few instances and several subjects complained of minor headaches either during or after the infusions. In only one subject (Subject K. C.), a control, was it necessary to terminate the infusion (after only 400 ml. had been given) because of severe nausea and retching.

As previously indicated, each amino acid infusion was preceded by a control saline infusion of the same amount (500 ml.) of physiologic (0.9 per cent) saline solution. This made it possible to estimate the base line excretion of amino acid plus the "washing out" effect produced by fluid containing no nitrogen. The amounts of α-amino acid found in the urine during this period were small. The average excretion for the control subjects was 5.7 mg. per hour with a range of 3.5 to 8.5 milligrams. The average excretion for the patients was 6 mg. per hour with a range of from 3 to 15 milligrams. There were no significant differences in the levels before or after operation.

The patterns of excretion of the amino acid mixture are shown in Fig. 1. Unfortunately, plasma amino nitrogen levels were not determined and our data do not permit the definite interpretation that the failure of the amino acids to appear in the urine indicates utilization rather than accumulation in the blood and altered renal clearance. The former assumption does, however, seem reasonable. The group of control subjects (Subjects B. P., W. J., and K. C.) thus showed an excellent "utilization" of the infused amino acids. Over half of the excretion measured took place during the infusion period. Three hours after the end of the infusion (four hours after the beginning), the urinary α-amino levels were only slightly above the control, preinfusion, levels. These results are similar to those of Eckhardt and Davidson[4] who found that the excess excretion of amino nitrogen above the control occurred within the first four hours following the infusion. They also report that the loss of amino nitrogen in a subsequent twenty-hour period was negligible and averaged 0.2 per cent of that injected.

In contrast to the rather uniform pattern of excretion found in the control subjects, the results of the preoperative control tests in the patient group were rather variable. In two of the patients (Patients L. C. and J. V.) the maximum excretion took place during the first hour after the infusion rather than during the infusion hour. The third patient (Patient B. R.) excreted approximately the same amount during the first two hours of the test. The fourth patient

showed a pattern similar to that of the controls. The total amounts excreted during the four-hour test indicated that these patients were "utilizing" the administered amino acids at least as effectively as did the control subjects.

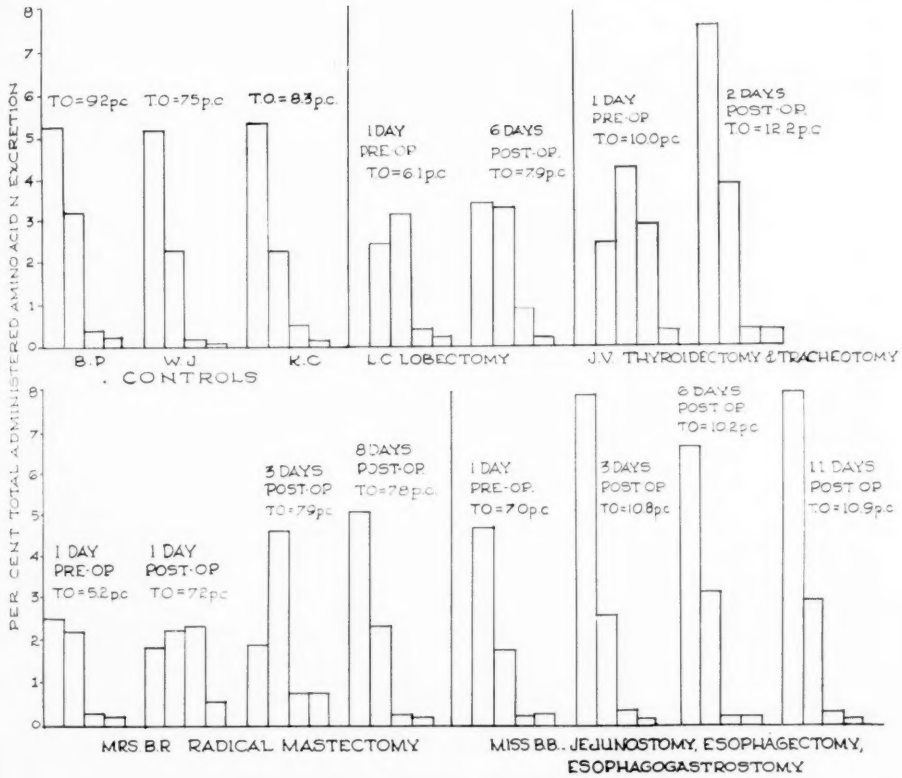

Fig. 1.—Alpha amino N excretion in control subjects and patients. *T. O.* = Total output of alpha amino N during the four-hour test period. Each block represents the amount excreted for an hour. Preinfusion levels are not given but the average amounts "washed out" during the control saline infusion period have been subtracted from the totals. Subject K. C. received only 400 c.c. of the amino acid mixture.

The data for caloric and nitrogen intake and for urinary nitrogen output are given in Table I. On the assumption that the average daily nitrogen output in the feces averaged 1.3 Gm., which for our present purposes seems reasonable, Patient L. C. was in positive nitrogen balance at the time of the first test before operation. Patients J. V. and B. R. were, likewise, in approximate equilibrium. The caloric and nitrogen intakes decreased markedly after operation as did the nitrogen balances. The nitrogen balances remained negative for about four days and rose as the food intake increased. The effect of the amino acid infusion on the urinary nitrogen could not be determined in Patient L. C. because of the failure to obtain twenty-four hour urine specimens on the days of the tests. In Patient J. V., the infusion was followed by an increase in urinary nitrogen which, however, was not sufficient to offset the favorable influence of the administered amino acid mixture on the nitrogen balance. Thus approximate nitrogen equilibrium was achieved on the second postoperative day in the face of a decreased

TABLE I

		INTAKE					OUTPUT	
			DIETARY N		INFUSION N		URINE N	
	DAY	CAL.	GM.	GM./SQ.M.	GM.	GM./SQ.M.	GM.	GM./SQ.M.
Patient L. C. (Wt., 68.0 Kg.; Ht., 173 cm.; S.A., 1.83 sq.M.)								
Preop.	5	2,550	13.9	7.60			12.8	7.00
	4	2,550	13.9	7.60			11.0	6.01
	3	2,550	13.9	7.60			10.4	5.13
	2	2,550	13.9	7.60			10.3	5.13
	1	1,600	6.9	3.77	7.35	4.0	---	---
Oper.	0	----	---	---			8.3	4.53
Postop.	1	162	1.3	0.11			---	---
	2	1,006	6.3	3.44			14.6	8.00
	3	1,333	9.0	4.92			15.0	8.20
	4	2,291	13.3	7.27			12.4	7.00
	5	2,137	12.7	7.00			13.1	7.15
	6	1,080	8.8	4.80	7.35	4.0	---	---
	7	3,097	12.1	6.61			9.8	5.36
Patient J. V. (Wt., 41 Kg.; Ht., 158 cm.; S.A., 1.36 sq.M.)								
Preop.	4	1,474	10.2	7.58			9.2	6.80
	3	1,474	10.2	7.58			9.1	6.70
	2	1,474	10.2	7.56			8.9	6.45
	1	1,258	7.8	6.00	7.35	5.4	11.1	8.16
Oper.	0	500	0.0	0.0			5.6	4.12
Postop.	1	83	0.7	0.52			7.1	5.22
	2	695	2.7	2.00	7.35	5.4	9.0	6.62
	3	1,556	6.3	4.63			7.4	5.44
	4	1,544	9.9	7.28			5.0	3.68
	5	1,638	11.4	8.40			8.0	6.00
	6	1,822	11.4	8.40	7.35	5.4	14.0	10.30
Patient B. R. (Wt., 66 Kg.; Ht., 158 cm.; S.A., 1.65 sq.M.)								
Preop.	4	1,690	12.0	7.27			9.8	6.00
	3	1,690	12.0	7.27			10.2	6.20
	2	1,690	12.0	7.27			11.2	7.00
	1	1,690	12.0	7.27			9.8	6.00
Oper.	0	----	---	---	7.35	4.45	10.9	6.61
Post.op.	1	----	---	---	7.35	4.45	16.0	9.70
	2	676	5.3	3.21			9.3	5.64
	3	766	5.5	3.33	7.35	4.45	13.3	8.05
	4	1,384	8.0	4.90			11.4	6.91
	5	1,502	9.3	5.64			8.7	4.70
	6	1,574	10.7	6.36			10.3	6.24
	7	1,684	11.0	6.67			10.8	6.54
	8	1,309	8.0	5.00	7.35	4.45	8.5	5.15

calorie and nitrogen intake. Patient B. R. was tested on the day of operation when there was no food intake. It may be assumed that the nitrogen balance would have been much more negative if the infusion had not been given.

The changes in the pattern of excretion of amino acids following surgery were variable. In each of the four cases, however, the percentage of the administered amino acid output rose and remained at a higher level than was observed preoperatively. In addition, irrespective of the preoperative patterns of excretion, the eventual postoperative pattern resembled those of control healthy subjects.

Fig. 2 illustrates the ratios of urinary urea, ammonia, and α-amino nitrogen excretion to the total urinary nitrogen excretion. Before the infusion of the

amino acid mixture, the distribution was essentially the same in the healthy controls and the pre- and postoperative tests on the patients. This agrees with previous observations that, following surgery, urea plus ammonia are excreted in their usual proportion.[2] The amino acid infusions resulted in an increase in amino acid excretion during the infusion period followed by a rapid return to the preinfusion pattern after the infusion was terminated. The results of the tests before and after operation do not show any significant alteration, except, perhaps, that the ratios of α-amino to total nitrogen excreted following the infusion tend to be higher after operation.

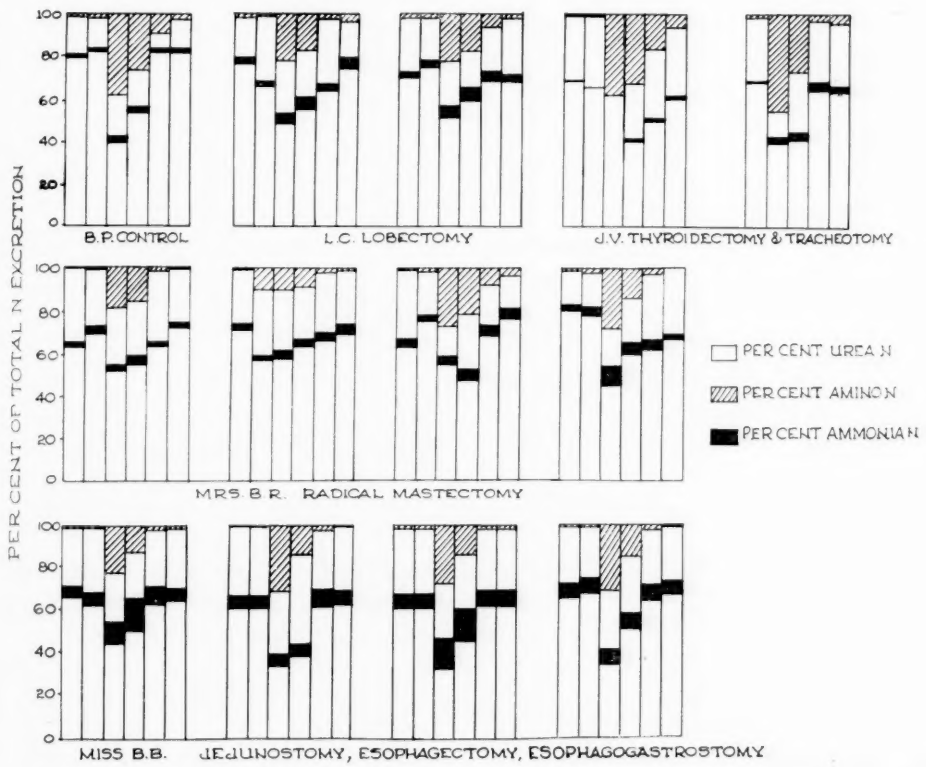

Fig. 2.—Ratios of urinary urea, ammonia, and alpha amino excretion. The first two blocks represent control hourly excretions during saline infusions. The remaining blocks are hourly values obtained after the amino acid infusion was given during the third hour. Patient B. R. received the amino acid infusion after only one hour of saline infusion on her second test. Data on Subject B. P. are given as representative of the results on the three control subjects.

Our experiments confirm and extend those of previous investigators[4, 5, 7] in showing that the rapid administration of amino acids intravenously in sick individuals, pre- and postoperatively, as well as in healthy subjects is followed by the immediate excretion of but a small fraction of that administered. The excretion of the amino acids is marked during the infusion and is practically completed at the end of four hours. The over-all excretion of α-amino acid during the test by the sick individuals was similar to or less than that of the control

healthy subjects, indicating at least as efficient a retention of the amino acids. The variations in the excretion patterns found in our patients, if they are significant, may be related to minor alterations in renal clearance rather than amino acid "utilization." As previously indicated, the eventual postoperative patterns always resembled those of control healthy subjects. It should be emphasized that our patients, postoperatively, were on inadequate caloric and nitrogen intakes at the time they showed negative nitrogen balances.

It is apparent that the capacity of the body to metabolize a large load of amino acids is not measurably altered in certain disease states and following surgery, at least under the conditions of our experiments. It might be of interest to know whether the additional "load" of a high protein diet would alter these observations.

SUMMARY

An amino acid mixture has been infused into three healthy subjects and into four patients before and after surgical operations.

Study of the nitrogen excretion confirms previous work indicating that most of the amino acid nitrogen is retained and that the major excretion occurs within four hours of the beginning of the infusion.

No significant differences in the amounts or patterns of amino nitrogen excretion were found after surgery when negative nitrogen balances were present.

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TURBIDIMETRIC GAMMA GLOBULIN DETERMINATIONS IN HEPATOBILIARY DISEASES

HANS POPPER, M.D., PH.D., J. DE LA HUERGA, M.D.,*
FREDERICK STEIGMANN, M.S., M.D., AND MOREY SLODKI, B.S.
CHICAGO, ILL.

IN PRECEDING studies[1] a simple turbidimetric test for serum gamma globulins was presented which correlates well with electrophoretic analysis.[2] Since flocculation and turbidity tests serve widely in the diagnosis of hepatobiliary diseases and since in many liver diseases the gamma globulins were found to be elevated by electrophoretic analysis,[3-12] the gamma globulin turbidity test was applied in a large number of patients with such diseases. Recently, the zinc sulfate turbidity test[13] has been widely used to estimate alterations in gamma globulin concentration. Although its results do not correlate as well with those of electrophoretic analysis for gamma globulins, it has proved to be of great diagnostic help in hepatobiliary diseases.[14-17] Therefore, the results obtained with the zinc sulfate turbidity test were compared with those of the gamma globulin turbidity test and the discrepancies found were explored.

MATERIAL AND METHOD

The gamma globulin turbidity test (GGT) and the zinc sulfate turbidity test (ZST) were performed in 227 serums of patients with various hepatobiliary diseases with and without jaundice. The diagnoses in these patients were made by clinical observations supported by serial laboratory determinations and the follow-up course, and in some instances these were confirmed by liver biopsy, operative and necropsy findings. In each patient a series of hepatic tests was performed. Cases with doubtful diagnosis were omitted. The findings in fourteen normal adults and 285 patients with diseases other than hepatobiliary were also considered for comparison. The results of the GGT were expressed in grams per 100 ml. (1.25 Gm. per 100 ml. was taken as the upper limit of normal while levels above 2.00 Gm. were considered a marked elevation). The results of the ZST were expressed in units (12.5 units was taken as the upper limit of normal and levels above 25 units were considered a marked elevation) or in grams of gamma globulins per 100 ml. calculated according to the conversion formula of Kunkel and co-workers.[14] The calibration curve of Shank and Hoagland[18] was used which is based on a 0.0962 normal barium chloride solution. Total serum bilirubin was determined according to Watson and Ducci;[19] cholesterol and cholesterol esters according to Chaney;[20] serum albumin by the method of Wolfson and associates;[21] and urinary urobilinogen in a two-hour sample according to Watson and co-workers.[22]

RESULTS

1. *GGT Test in Various Hepatobiliary Diseases.*—The most marked elevation of the GGT was found in patients with cirrhosis with jaundice (Table I). It was absent in only a few instances. It exceeded 2 Gm. in almost two-thirds

From the Hektoen Institute for Medical Research and the Departments of Pathology and Therapeutics of Cook County Hospital; the Department of Pathology of Northwestern University Medical School; and the Department of Internal Medicine, University of Illinois College of Medicine.

Supported by a grant from the Dr. Jerome D. Solomon Memorial Research Foundation.
Received for publication, Dec. 1, 1949.

*Solomon Fellow.

TABLE I. RESULTS OF GAMMA GLOBULIN TURBIDITY AND ZINC SULFATE TURBIDITY TESTS AS WELL AS ZINC SULFATE TURBIDITY/GAMMA GLOBULIN TURBIDITY RATIO IN HEPATOBILIARY DISEASES

		GAMMA GLOBULIN TURBIDITY				ZINC SULFATE TURBIDITY				
				PERCENTAGE INCIDENCE				PERCENTAGE INCIDENCE		
DISEASE	NUMBER OF CASES	MEAN (GM. %)	D.S.	ABNORMAL RESULTS	RESULTS ABOVE 2 GM. %	MEAN (UNITS)	D.S.	ABNORMAL RESULTS	RESULTS ABOVE 2 GM. %	MEAN RATIO ZST/GGT
Severe viral hepatitis	38	1.71	0.53	87	24	18.8	8.9	84	21	10.7
Homologous serum hepatitis	11	2.01	0.39	100	46	22.3	8.1	82	27	10.8
Infectious hepatitis	27	1.59	0.36	77	15	16.7	7.5	82	15	10.7
Mild viral hepatitis	14	1.45	0.32	79	0	17.8	4.8	64	7	10.5
Recovered viral hepatitis	7	1.50	0.26	71	0	13.3	3.9	71	0	8.7
Severe toxic hepatitis	17	1.67	0.61	76	23	15.6	8.7	53	18	9.7
Mild toxic hepatitis	9	1.64	0.58	77	22	16.5	10.2	44	22	9.5
Cirrhosis with jaundice	59	2.32	0.72	92	60	25.7	12.2	86	42	10.5
Cirrhosis without jaundice	37	1.82	0.53	88	43	17.6	7.7	67	16	9.4
Obstructive jaundice	26	1.43	0.44	68	11	9.8	5.8	27	4	6.7

of the patients. In cirrhosis without jaundice (58 per cent of which revealed ascites and 52 per cent of which showed abnormal cephalin flocculation) the average was somewhat lower but in less than one-half of the cases were values above 2 Gm. found. In viral hepatitis, as a whole, the gamma globulin elevation was lower than in cirrhosis. While very few patients had normal levels, only one-fourth had levels above 2 grams. In mild viral hepatitis the average was

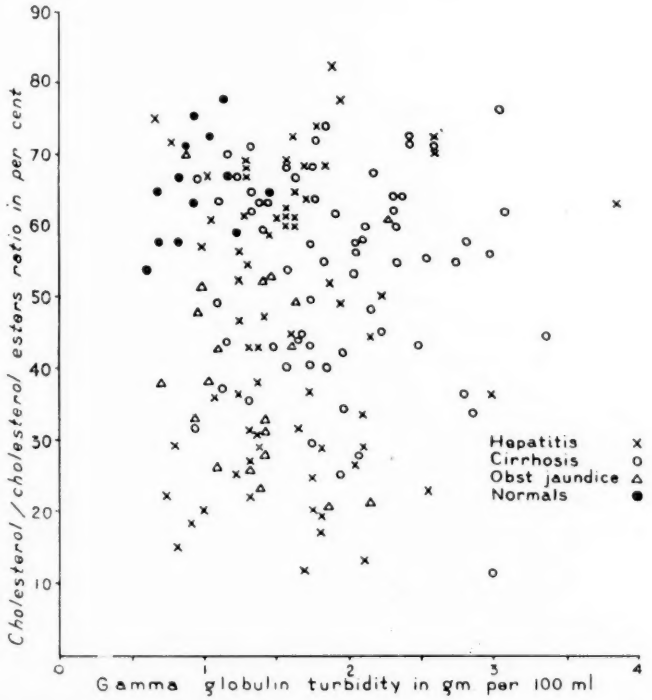

Fig. 1.—Plot of gamma globulin turbidity against cholesterol esters/cholesterol ratio in patients with various hepatobiliary disorders.

significantly above the normal with great regularity. This persisted even in the clinically recovered stages when all other tests yielded normal results and jaundice had subsided. Toxic hepatitis, either mild or severe, did not differ significantly from viral hepatitis. In extrahepatic obstructive jaundice (due to stones, tumors, scars), the GGT was slightly elevated and was normal only in one-third of the cases. In a small percentage, it even rose above 2 Gm. per 100 milliliters. A correlation with duration of jaundice and presence of infection was not apparent.

2. *Relation of GGT to Serum Bilirubin and Cholesterol Esters/Cholesterol Ratio.*—The GGT level was plotted against the total serum bilirubin and the cholesterol esters/cholesterol ratio (Fig. 1). This was done to investigate a possible relation between gamma globulin elevation and the degree of jaundice or liver cell damage in general or in individual diseases. However, no correlation could be demonstrated.

3. *Comparison Between the Homologous Serum and the Infectious Types of Viral Hepatitis.*—The thirty-eight cases of severe viral hepatitis were divided into those in which blood and plasma transfusions or extensive intravenous medication two to eight months prior to the outbreak of jaundice suggested a homologous serum type (eleven cases) and into the naturally occurring form (infectious hepatitis) (twenty-seven cases). The GGT was on the average higher in homologous serum hepatitis, but the difference was not statistically significant.

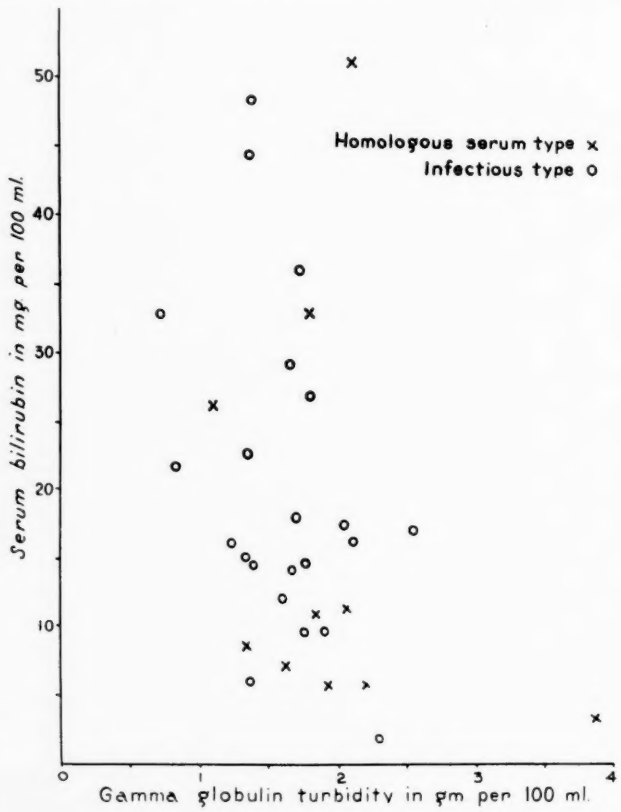

Fig. 2.—Plot of gamma globulin turbidity against serum bilirubin concentration in patients with severe viral hepatitis.

However, all cases of homologous serum hepatitis revealed an elevation of gamma globulins and in almost half of them it exceeded 2 Gm. per 100 milliliters. To investigate the possibility that this suggested difference may be an expression of greater severity rather than one of a difference in the character of the disease, the total serum bilirubin level (Fig. 2) as well as the cholesterol esters/cholesterol ratio were plotted against the gamma globulin concentration. However, no correlation was found in that patients with homologous serum hepatitis may show, despite high gamma globulins, a low bilirubin concentration and a high cholesterol esters ratio, and vice versa in infectious hepatitis.

4. *Transition of Hepatitis Into Cirrhosis.*—In eight of the cases of acute hepatitis studied, a tendency of transition into cirrhosis was apparent from

either biopsy or clinical observations. The GGT was high in almost all instances, the mean equaling that of cirrhosis with jaundice (Table II). The ZST was also elevated but not quite as markedly. The thymol turbidity, cephalin flocculation, and Takata-Ara tests were moderately, if at all, elevated. The cholesterol esters/cholesterol ratio was not necessarily abnormal and the total serum bilirubin revealed wide variations. A patient in whom the examinations were repeated at an interval of forty days showed a simultaneous elevation of GGT

TABLE II. RESULTS OF HEPATIC TESTS IN EIGHT CASES OF VIRAL HEPATITIS REVEALING TRANSITION INTO CIRRHOSIS

	MEAN	RANGE
Gamma globulin turbidity (Gm. %)	2.20	1.61-3.10
Zinc sulfate turbidity (units)	25.4	13.2-38.8
Cephalin flocculation	2.4+	0-4+
Thymol turbidity (units)	8.3	2-15
Takata-Ara test	2.1+	1-4+
Total serum bilirubin (mg. %)	11.4	1.0-32.0
Cholesterol esters/cholesterol ratio (%)	52	31-74

and ZST while the total serum bilirubin and cephalin flocculation dropped and the cholesterol esters/cholesterol ratio rose to normal. Thymol turbidity and Takata-Ara test failed to reveal significant changes (Table III).

5. *ZST in Various Hepatobiliary Disorders.*—In general, the ZST paralleled the GGT with several exceptions (Table I). The elevation of the ZST in cirrhosis with jaundice was not as marked as that of the GGT. Thus, less than one-half of the patients showed an elevation above 25 units. In cirrhosis without

TABLE III. CHANGES IN RESULTS OF HEPATIC TESTS WITHIN 40 DAYS IN A REPRESENTATIVE CASE OF HEPATITIS REVEALING TRANSITION INTO CIRRHOSIS

(A 40-year-old colored woman developed icterus during the sixth week of antisyphilitic therapy. She was severely icteric, the liver was slightly enlarged, the spleen was of normal size. She improved slowly with a decrease in liver size and disappearance of jaundice within 4 weeks.)

	FROM	TO
Gamma globulin turbidity (Gm. %)	1.40	2.65
Zinc sulfate turbidity (units)	14.2	33.0
Cephalin flocculation	3+	0
Thymol turbidity (units)	4.6	5.2
Takata-Ara test	1+	2+
Total serum bilirubin (mg. %)	28	2
Cholesterol esters/cholesterol ratio (%)	43	71

jaundice the mean of the ZST was even lower than in severe viral hepatitis, and elevated levels were found in only two-thirds of the cases. In recovered viral hepatitis, the mean ZST was slightly above the upper limit of normal. The difference between mild viral and recovered viral hepatitis was pronounced. In contrast, the mean of the ZST was decidedly lower in obstructive jaundice than in the various intrahepatic lesions. It was above normal in only one-fourth of the cases, again without detectable correlation with the clinical picture.

6. *Ratio of ZST/GGT in Various Conditions.*—The demonstrated difference between ZST and GGT is exemplified by the ratio between ZST in units and GGT in grams per 100 ml. (Table I). This ratio averaged around 10 in most of

the hepatobiliary conditions studied; it was lower in recovered viral hepatitis and especially so in obstructive jaundice indicating that in the latter the ZST is much lower than the gamma globulin concentration would indicate. A plot of the ZST against the GGT (Fig. 3) indicated again a tendency of the ZST to be

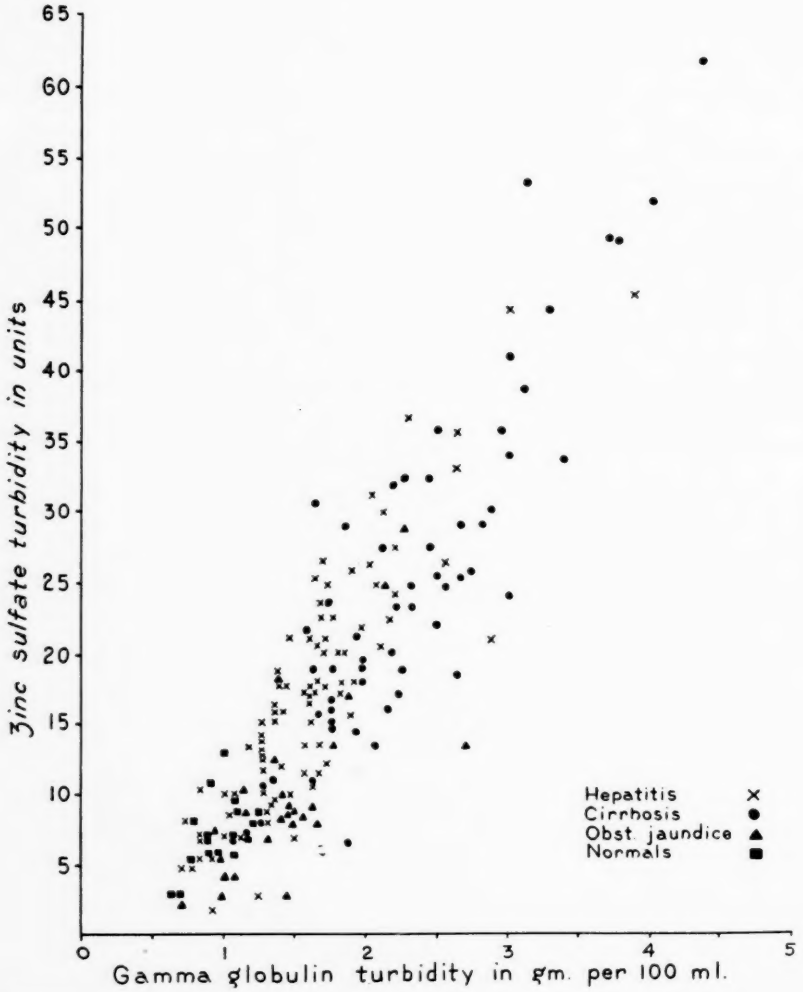

Fig. 3.—Plot of gamma globulin turbidity against zinc sulfate turbidity in patients with acute hepatitis, cirrhosis with jaundice, and extrahepatic obstructive jaundice and in normal subjects.

lower in comparison with the GGT in obstructive jaundice than would be expected from the gamma globulin level. This tendency was less marked in normal subjects in which the GGT was equally low.

7. *Analysis of the Factors Causing Depression of ZST in Obstructive Jaundice.—*

(a) Influence of Whole Bile on ZST: The addition of human gall bladder bile to serum so as to increase its bilirubin concentration to levels commonly en-

TABLE IV. INFLUENCE OF ADDITION OF WHOLE BILE AND LECITHIN TO SERUM UPON THE ZINC SULFATE AND GAMMA GLOBULIN TURBIDITY; THE DILUTION OF THE SERUM IS TAKEN INTO ACCOUNT

	SERUM	1 ML. SERUM PLUS 0.02 ML. BILE (BILIRUBIN: 11 MG. %)	1 ML. SERUM PLUS 0.04 ML. BILE (BILIRUBIN: 22 MG. %)	1 ML. SERUM PLUS 0.2 ML. 6% LECITHIN (INCREASE IN LECITHIN: 1,000 MG. %)
Serum I				
ZST (units)	23.0	18.4	17.8	18.0
GGT (Gm. %)	2.12	2.10	2.19	2.05
Serum II				
ZST (units)	17.6	16.4	15.0	16.0
GGT (Gm. %)	1.63	1.64	1.72	1.66
Serum III				
ZST (units)	9.0	7.7	6.4	8.0
GGT (Gm. %)	1.08	1.08	1.13	1.08

countered in obstructive jaundice proportionally reduced the ZST without influencing the GGT (Table IV). Similarly, the addition of lecithin to serum in amounts encountered in obstructive jaundice depressed the ZST without influencing the GGT.

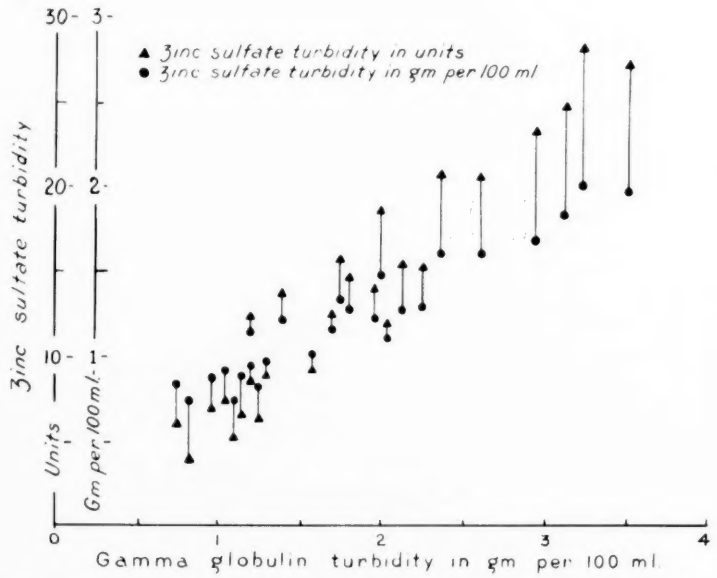

Fig. 4.—Plot of gamma globulin turbidity against zinc sulfate turbidity, the latter being expressed in grams per 100 ml. and in units. The latter two values of each case are connected by a line.

(b) Influence of Unitage: As mentioned in a preceding paper,[2] the correlation between ZST and gamma globulin concentration as determined electrophoretically is different if the ZST is expressed in grams per 100 ml. instead of in units. In the higher concentrations of gamma globulin, the correlation with electrophoretic results was better when the results were expressed in units, while at lower concentrations it was better when expressed as grams per 100 milliliters. Kunkel's[13] results showed a tendency of the unit values to drop

markedly in lower concentrations. This factor is taken into account in his conversion formula;[14] however, the latter does not allow for various heights of the gamma globulin levels. If the ZST was expressed in the same cases in both units and grams per 100 ml. and both values were plotted against the GGT (as was done for a series of randomly selected cases with different concentration of gamma globulins, Fig. 4), the unit values were lower than the values in grams in the lower range and higher in the abnormal range. Thus, the low levels of the ZST in obstructive jaundice are partially explained by this tendency of the ZST units to deviate from a proportionate relationship with the gamma globulin concentration in the low ranges.

DISCUSSION

The turbidimetric estimations of the serum gamma globulin concentration in a large number of cases confirmed observations obtained previously by the electrophoretic method. Gamma globulin elevation (checked electrophoretically in half of the cases studied[23]) is found in all hepatic disorders and is most marked in chronic conditions such as cirrhosis,[3, 4, 6, 9, 10] especially when jaundice indicates a superimposed acute process. In acute hepatitis, also, a considerable elevation is noted [4, 5, 7, 8] which does not completely disappear in the recovery period. The elevation is slight only in the obstructive form of jaundice where it barely exceeds the upper limit of the normal.

These experiences have to be considered from two angles: (1) the basic significance of the gamma globulin elevation, and (2) the diagnostic value of the simple test. Although it appears established that albumin is formed by the liver cells, the origin of the globulins is questionable.[24] Some of the alpha globulins are produced by the liver cells[25] as are specific globulins such as prothrombin. However, all available evidence indicates that gamma globulin is formed by reticuloendothelial[26] and plasma cells[27, 28] or lymphocytes.[29, 30] Applied to the liver, this would point to production by the Kupffer cells and possibly also by mesenchymal cells in the portal triads. Gamma globulin elevation in liver disease is therefore not to be considered the direct result of liver cell damage but rather of a stimulation of the Kupffer cells and other mesenchymal cells in the liver itself or reticuloendothelial cells in other organs by breakdown products of liver cells. Histologically, the stimulation of the Kupffer cells is indicated by their mobilization. In the cytoplasm of the mobilized Kupffer cells ribose nucleic acid becomes demonstrable[31] and is probably related to protein formation.[32, 33] Its appearance in the Kupffer cells can thus be considered a morphologic sign of increased gamma globulin production similar to that seen in lymphatic elements after immunization.[28, 30] It has been shown[6] that the increase in gamma globulin is not merely a result of infection nor a compensatory mechanism for the hypoalbuminemia in contrast to previously stated opinions.[4] Whether the increased gamma globulin production associated with the mesenchymal reaction is an expression of antibody formation is questionable although antibodies have been described in hepatitis.[34, 35] The excess gamma globulin is, however, probably a nonspecific product of stimulated reticuloendothelial cells (reaction globulin[12]) for which antibody formation may represent

the initial stimulus. In cirrhosis where the gamma globulin elevation is most marked, the fibroblasts are stimulated, probably also by liver cell breakdown products. Possibly both mesenchymal reactions, i.e., excessive gamma globulin production and fibroplasia, are the expression of the same process. This may be analogous to the parallel gamma globulin elevation and changes of the connective tissue seen in collagen diseases in which recently both processes have been considered one unit.[36]

The assumption that the gamma globulin elevation is related to the mesenchymal reaction rather than to the liver cell damage agrees with the relatively low gamma globulin levels in obstructive jaundice. In this condition, the Kupffer cells, even if proliferated, are loaded with bile pigments and no ribose nucleic acid can be demonstrated in their cytoplasm.[31] This suggests that excessive jaundice suppresses the characteristic chemical response of the Kupffer cells.

Chronic hepatitis with transition into cirrhosis shows an elevation of the gamma globulin at a time when the liver function is improving, the jaundice is decreasing, and cephalin flocculation and thymol turbidity have become normal. Whether this increase is an expression of antibody formation or of a nonspecific mesenchymal response to liver cell breakdown product is again questionable. A similar problem is presented by the fact that the gamma globulin levels are often higher in homologous serum in contrast to the naturally occurring infectious hepatitis. The difference is not due to the known[37, 38] severity of the disease as measured by the degree of jaundice or the cholesterol esters/cholesterol ratio (the latter as index of liver cell damage). However, not too much emphasis can be given to this difference since the number of cases examined is relatively small and the differentiation of the two forms of viral hepatitis from the history is not reliable.

In discussing the practical use of the GGT, it must be compared with the ZST. Elevation of gamma globulins is of limited diagnostic value in hepatobiliary diseases because it may occur in many conditions associated with chronic infection.[39] But if a chronic infection or other nonhepatobiliary disease can be excluded on clinical grounds, the gamma globulin elevation may be of diagnostic significance for the recognition of cirrhosis or the transition of acute hepatitis into cirrhosis. It may be valuable for recognition of hepatitis, especially when serially performed. In all these circumstances, the elevation of the GGT is more marked and occurs more regularly than that of the ZST. However, for the differentiation of obstructive surgical jaundice from the medical variety, the GGT is of no diagnostic significance. On the other hand, in this practical point, the ZST is of great value as has been pointed out repeatedly.[14-17] Several factors are responsible for the fact that the ZST, which otherwise correlates well with the GGT, is low in obstructive jaundice. One is the depression of the ZST by substances in the bile possibly of lipid character such as lecithin. Second, a normal serum albumin (which is usually not found in cirrhosis or hepatitis) has a depressive effect upon the ZST. Third, there is a mathematical factor which is based on the tendency of the units of the ZST to be much lower in the normal and low ranges that the gamma globulin concentration would

indicate. This tendency is an expression of a relatively much smaller precipitate in the lower range. It is also found with the ammonium sulfate-sodium chloride precipitate of the GGT test but was mathematically compensated for either by the proposed conversion formula or by calibration of the standard curve with serums of known gamma globulin concentration. The conversion formula of ZST units to grams given by Kunkel[14] corrects for this tendency. However, it causes an error in the high ranges where it produces too low values. This discrepancy between ZST units and GGT grams, which is characteristic of all conditions with low gamma globulins and not only of obstructive jaundice, could be compensated for by a better conversion formula. However, expression of the ZST in units, combined with the depressing factor of albumin and of bile, emphasizes the differences between obstructive jaundice and primary hepatitis. For this purpose, therefore, the ZST in units, although it does not mirror gamma globulin concentration and produces a mathematical illusion, is superior to the GGT.

The ratio between ZST and GGT may have some diagnostic significance. It is, as a rule, lower in the surgical type of jaundice (extrahepatic biliary obstruction due to tumors, scars, stones) than in the medical type (hepatitis and cirrhosis). The interference with the bile flow itself is probably the most important factor as it produces regurgitation of biliary substances into the blood; in the stage of complete obstruction in both types of jaundice the ratio is significantly lower, especially so in the extrahepatic form.

SUMMARY

1. The gamma globulin turbidity test (GGT) was performed simultaneously with the zinc sulfate turbidity test (ZST) in 207 patients with various hepatobiliary diseases. Gamma globulin elevation of different degrees was found in all types of hepatobiliary diseases studied. It was associated with the mesenchymal reaction characteristic of the various hepatic disorders which is expressed by proliferation of the Kupffer cells and histiocytes in the portal triads and which parallels the fibroplasia leading to cirrhosis formation. The gamma globulin elevation did not show any relation to the degree of jaundice nor to the cholesterol esters/cholesterol ratio (as an estimate of liver cell damage).

2. The gamma globulin elevation is very marked in cirrhosis, especially when jaundice is present. It is less marked in acute toxic and infectious hepatitis but may persist in recovery when the other tests are already normal. A gamma globulin increase during the recovery period of hepatitis points to a transition into cirrhosis. In all the conditions listed, the ZST reveals less consistent results.

3. In extrahepatic obstructive jaundice the GGT is as a rule slightly elevated whereas the ZST is below the upper limit of the normal. Therefore, only the latter may be useful in the differential diagnosis between surgical and medical jaundice.

4. The relatively low ZST in obstructive jaundice is explained by the interplay of several depressing factors. Two are found in all conditions with low gamma globulins: (a) the tendency of the ZST, as expressed in units, to deviate

in the low range from a proportional relationship with the gamma globulins, and (b) the normal albumin level which counteracts the ZST. The other factor occurring primarily in obstructive jaundice is a depression of the ZST by regurgitated biliary substances, probably lipid in character.

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OBSERVATIONS ON THE DIAGNOSTIC VALUE OF LIVER BIOPSY, TESTS OF HEPATIC FUNCTION, AND ELECTROPHORETIC FRACTIONATION OF SERUM PROTEINS IN ASYMPTOMATIC PORTAL CIRRHOSIS

WILLIAM E. RICKETTS, M.D., JOSEPH B. KIRSNER, M.D.,
WALTER L. PALMER, M.D., AND KENNETH STERLING, M.D.
CHICAGO, ILL.

MANY patients with portal cirrhosis never develop symptoms; the process may be recognized only at autopsy, death occurring from causes unrelated to the liver. Other patients may experience hepatic failure and achieve complete clinical recovery. The purpose of this paper is to analyze the results of punch biopsy of the liver, tests of hepatic function, and the electrophoretic pattern of the serum proteins in a representative group of patients with asymptomatic cirrhosis, and to evaluate their diagnostic significance.

The presence of cirrhosis initially was suggested by the demonstration, on routine physical examination, of splenomegaly, hepatomegaly, collateral circulation, telangiectasis, or esophageal varices. The clinical features are discussed in a separate publication.[1]

METHOD OF STUDY AND RESULTS

(a) Liver Biopsy.—Twenty cases of portal cirrhosis were studied. The diagnosis was established conclusively in nineteen by punch liver biopsy. In one patient the histologic examination of hepatic tissue obtained by this procedure disclosed normal findings; however, operation for splenectomy revealed an extensive cirrhosis with irregular nodulations of regenerated liver separated by wide areas of scarring. Varying degrees of hepatic fibrosis were found histologically in all patients; four typical sections are shown in Fig. 1.

*(b) Tests of Hepatic Function.**—A series of hepatic function tests was obtained simultaneously using techniques described in a previous publication.[2] Twenty normal individuals served as controls (Table I). The first thirteen subjects had been entirely asymptomatic.

The last seven subjects had been severely ill with hepatic insufficiency; six had had ascites and had required repeated paracenteses; one patient had been comatose for a period of three days. The present study was made two to five years after recovery. The observations may be summarized as follows.

Serum Bilirubin: An elevated serum bilirubin was found in only one patient, the values being a direct reaction of 0.4 and a total of 1.3 mg. per cent. All other cases fell within normal range.

Cholesterol: The total serum cholesterol was determined in seventeen patients. In four the values were between 300 and 350 mg. per cent; the remainder were within the normal range. The cholesterol-cholesterol ester ratio was normal in the fourteen determinations made.

From the Frank Billings Medical Clinic, Department of Medicine, University of Chicago.

This investigation has been made with the assistance of a grant from the Committee on Therapeutic Research, Council of Pharmacy and Chemistry of the American Medical Association.

Received for publication, Dec. 9, 1949.

*Many of these procedures do not measure hepatic function per se.

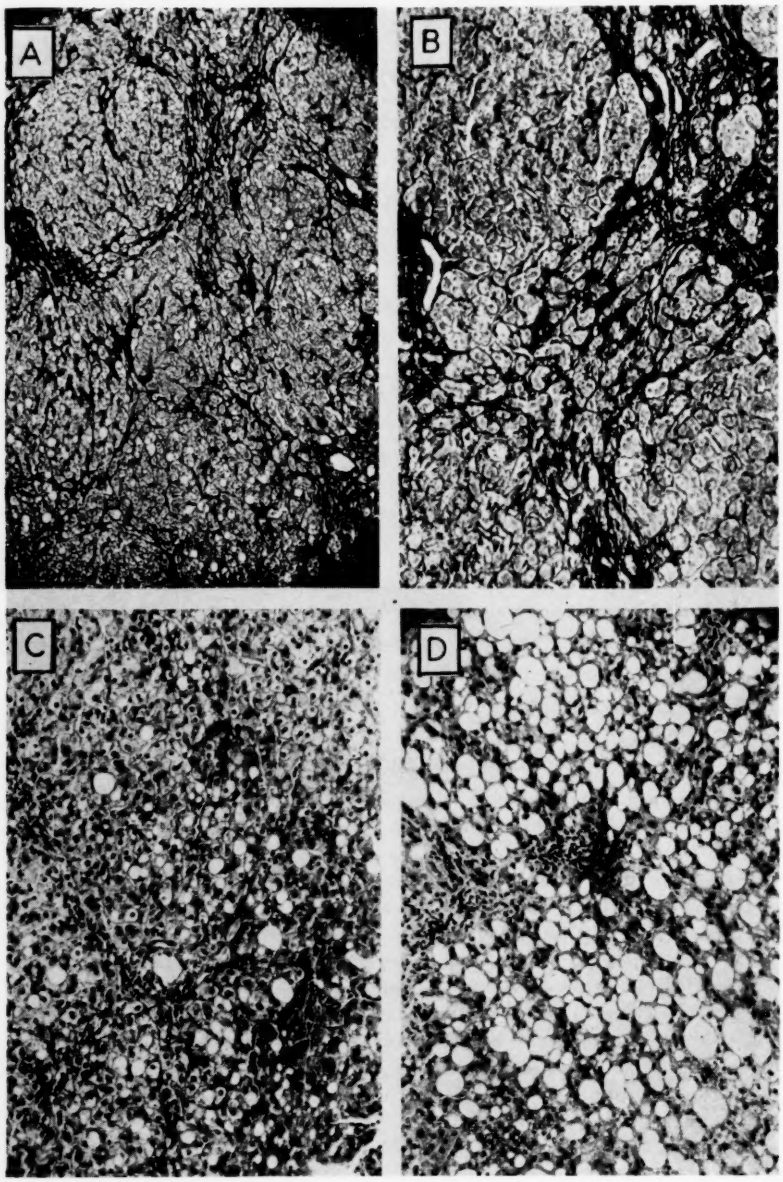

Fig. 1.—Photomicrographs of sections from asymptomatic untreated patients with portal cirrhosis. Punch liver biopsies disclosing marked fibrosis in *A* and *B* and fatty degeneration of the liver in *C* and *D*.

Plasma Proteins: The total plasma proteins varied, but never were less than 6 Gm. per cent. Increased total proteins were noted in several instances, the highest being 9.08 Gm. per cent; the higher values usually were due to an increase in the globulin fraction. A plasma albumin below 4 Gm. per cent (3.79 Gm. per cent) was noted in only one case. Elevation of the plasma globulin above 3 Gm. per cent occurred in four instances, the highest being 4.26 Gm. per cent.

TABLE I. TESTS OF HEPATIC FUNCTION

SUBJECT	BILIRUBIN (MG. PER CENT)		CHOLESTEROL (MG. PER CENT)		PROTEINS (GM. PER CENT)			ALKALINE PHOSPHATASE (BODANSKY UNITS)	THYMOL TURBIDITY (UNITS)	CEPHALIN-CHOLESTEROL FLOCCULATION	HIPPURIC ACID (GRAMS)	PROTHROMBIN TIME (PER CENT)	URINARY UROBILINOGEN (MG./24 HR.)	B.S.P. (PER CENT)
	DIRECT	TOTAL	TOTAL	ESTERS	ALBUMIN	GLOBULIN	TOTAL							
1	.4	.7	205	135	4.41	2.86	7.27	4.0	9.0	Neg.	1.3	---	0.18	12.0
2	.3	.7	285	---	4.76	2.18	6.96	5.9	3.0	++	0.1	---	3.19	5.0
3	.2	.4	320	240	4.65	2.84	7.49	4.1	0.9	+	---	92	---	15.0
4	.3	.5	302	215	4.87	2.55	7.42	3.0	8.2	+	0.3	87	2.07	28.0
5	.5	1.0	225	150	4.73	2.75	7.48	6.7	3.8	Neg.	0.4	100	11.6	1.0
6	.3	.7	325	235	4.61	2.55	7.16	3.5	2.8	+	1.4	92	0.05	16.0
7	.2	.4	200	142	4.21	3.28	7.49	2.8	2.0	Neg.	---	100	1.0	22.0
8	.2	.6	235	188	4.66	2.53	7.19	4.7	2.9	Neg.	0.7	101	0.32	2.5
9	.7	1.3	328	---	4.50	4.16	8.66	2.6	13.0	+	0.6	97	---	15.0
10	.2	.6	---	---	4.82	4.26	9.08	4.9	2.5	+++	---	---	---	5.0
11	.2	.6	---	---	4.75	2.48	7.23	5.7	4.5	Neg.	---	90	0.62	38.0
12	.4	.9	134	104	4.34	3.50	7.84	4.8	7.3	+++	0.9	94	0.20	60.0
13	.3	.7	188	133	4.10	2.12	6.32	6.4	3.6	+++	---	85	0.83	100.0
14	.2	.6	---	---	3.79	2.97	6.76	4.0	5.8	+	---	96	2.49	15.0
15	.2	.5	215	150	4.06	2.80	6.86	4.7	1.2	++	1.2	91	0.5	6.5
16	.3	.6	245	170	4.29	1.57	5.86	2.2	9.8	+++	1.4	84	0.6	13.0
17	.2	.6	245	175	4.62	2.62	7.24	5.9	7.6	+	0.9	90	0.38	34.0
18	.3	.5	250	184	4.88	1.98	6.86	4.3	4.2	Neg.	1.3	90	0.53	8.0
19	.0	.2	245	181	5.22	2.25	7.47	3.7	4.3	++	1.3	97	1.15	8.0
20	.1	.6	245	182	4.46	1.67	6.13	2.0	1.3	Neg.	1.4	96	0.4	3.5
NORMAL SERA														
Average	0.20	0.56	224.6	74.0	4.81	2.18	7.02	3.43	<5.0	<++	1.08	94.3	<3	<10
Standard deviation	0.13	0.21	28.8	6.95	0.34	0.35	0.31	1.03			0.34	10.6		

Alkaline Phosphatase: Increases in the alkaline phosphatase above 5 Bodansky units were observed in only four patients; none of the values exceeded 7 units.

Flocculation Tests: The results of the thymol turbidity and cephalin-cholesterol flocculation tests were extremely variable. Thymol turbidity values below 5 units were noted in twelve patients; definitely abnormal values were obtained in eight patients, the highest being 13 units. A cephalin flocculation of plus 3 was obtained in four patients, plus 2 in two patients; the test was negative in the remaining patients.

Hippuric Acid Excretion: Elimination of hippuric acid of less than 0.7 Gm. per cent was obtained in only four out of twelve patients tested.

Prothrombin Time: The prothrombin time was below 85 per cent of normal in only one patient.

Urobilinogen Excretion: The urinary urobilinogen exceeded 3 mg. per cent for 24 hours in only two cases; the highest value was 11 milligrams. The fecal urobilinogen in all patients tested was within the normal range.

Bromsulfalein Retention: Bromsulfalein retention proved to be the best screening procedure in that a retention of 10 per cent or more after 30 minutes was found in 65 per cent of the cases.

(c) Electrophoretic Data.—The electrophoretic patterns of the plasma proteins were studied in nine patients, using methods described in a previous publication[3] (Table II). Three (Subjects J. C., M. M., and D. B.) were untreated asymptomatic patients, and six (Subjects J. H., R. S., G. O., C. R., A. E., and W. G.) had been observed in severe parenchymal failure but had recovered on medical management and had been free of symptoms for periods of two to five years. Slightly abnormal patterns with elevation of gamma or beta globulins were observed in some instances; these changes were not constant; however, in some patients the patterns were entirely similar to those of normal individuals.

TABLE II

	ELECTROPHORETIC DATA											HOWE SODIUM SULFATE			
	PER CENT					GRAMS PER CENT							GRAMS PER CENT		
SUBJECT	ALB.	α_1	α_2	β	γ	ALB.	α_1	α_2	β	γ	A/G	A/G	ALB.	GLOB.	TOT.
J. C.	55.1	5.9	14.3	13.7	11.0	3.61	0.39	0.94	0.90	0.72	1.23	1.07	3.41	3.15	6.56
M. M.	52.2	4.0	10.4	18.6	14.8	4.14	0.32	0.83	1.47	1.17	1.09	1.61	4.89	3.04	7.93
D. B.	55.2	4.5	10.3	17.8	12.2	3.99	0.33	0.74	1.29	0.88	1.23	1.91	4.75	2.48	7.23
J. H.	56.4	3.5	9.6	15.8	14.7	3.87	0.24	0.66	1.08	1.01	1.29	1.45	4.06	2.80	6.86
R. S.	58.0	4.3	8.4	14.5	14.8	3.90	0.29	0.56	0.98	1.00	1.38	1.85	4.37	2.36	6.73
G. O.	55.9	5.4	8.4	14.9	15.4	4.05	0.39	0.61	1.08	1.11	1.27	1.76	4.62	2.62	7.24
C. R.	55.2	5.4	10.2	16.2	13.0	3.84	0.38	0.71	1.12	0.90	1.23	1.74	4.41	2.54	6.95
A. E.	62.6	4.0	8.2	16.8	8.4	4.13	0.26	0.54	1.11	0.55	1.68	2.62	4.77	1.82	6.59
W. G.	59.4	3.5	13.1	14.4	9.6	4.36	0.26	0.96	1.06	0.70	1.46	2.07	4.95	2.39	7.34
NORMAL SERA															
Average	60.3	4.0	9.7	12.8	13.2	4.23	0.28	0.68	0.89	0.93	1.52	2.31	4.89	2.12	7.01
Standard deviation	2.8	2.8	1.5	1.2	1.7	0.29	0.06	0.10	0.08	0.13	0.18	0.34	0.30	0.24	0.30

DISCUSSION

It is of obvious clinical importance to establish the diagnosis of portal cirrhosis conclusively. In a previous report,[1] asymptomatic portal cirrhosis constituted approximately one-third of the total number of cases of portal cirrhosis. This figure is considerably lower than the incidence of so-called latent cirrhosis as found at necropsy.[4] The present study indicates that the

determination of direct and total bilirubin, total serum cholesterol and cholesterol-cholesterol ester ratio, alkaline phosphatase, thymol turbidity, cephalin-cholesterol flocculation, hippuric acid elimination, and the quantitative measurement of urinary urobilinogen are of little value in this respect. The best screening test is the bromsulfalein excretion, confirming a previous observation.[5] Apparently the removal of bromsulfalein from the organism involves a dual mechanism. Wirts and Cantarow[6] demonstrated an initial phase with rapid removal of the dye from the blood stream, depending largely on the activity of the Kupffer cells, and a second phase with gradual release and excretion of the dye by the polygonal cells of the liver.

In a recent publication[7] the alterations of function in portal cirrhosis have been shown to be related to changes in the parenchyma rather than to the hepatic fibrosis, and furthermore, it has been shown that these alterations often are reversible. Thus, the results of the present study are not surprising, for in asymptomatic cirrhosis the alterations in the polygonal parenchymal cells are minimal.[7]

The value of punch liver biopsy in the diagnosis of the asymptomatic cases of portal cirrhosis is conclusively established. Of considerable interest was the observation of a patient in whom punch liver biopsy disclosed bands of deep scarring separated by areas of apparently normal liver tissue; the biopsy needle had entered one of these areas. It may be noted that in some patients clinically suspected of having portal cirrhosis, needle biopsy has disclosed hepatic disease other than cirrhosis. Nevertheless, in the recognition of clinically asymptomatic portal cirrhosis, needle biopsy of the liver, together with the bromsulfalein excretion test, provides the most useful objective evidence of the disease.

CONCLUSIONS

In asymptomatic or latent portal cirrhosis:

1. Present tests of hepatic function are rather unreliable. Bromsulfalein excretion seems to be the best screening procedure.

2. Electrophoretic fractionation of the serum proteins does not disclose characteristic patterns.

3. Punch needle biopsy of the liver is an excellent but not an infallible diagnostic procedure.

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A SERUM POLYSACCHARIDE IN CANCER

FERDINAND G. WEISBROD, M.Sc., M.D.*
CINCINNATI, OHIO

UTILIZING a test for the detection of a serum polysaccharide, Seibert[1] has shown a significant increase in this component in cases of tuberculosis as well as in carcinoma. Although the exact nature of the test substance is not known, desoxyribonucleic acid derived from the breakdown of nucleoproteins and thus reflecting tissue destruction may be implicated.[1, 2] This report represents an attempt to confirm Seibert's work in regard to neoplastic disease and to determine the clinical significance in such patients.

METHOD

Seibert's method[1] was modified for use with the Coleman Junior spectrophotometer instead of the Klett colorimeter.

Reagents.—

Perchloric acid: C.P. 60 per cent.

Saline: 0.9 per cent in distilled water.

Tryptophan: 0.25 per cent of the purest product available. The crystals were put into solution with 0.075N NaOH (20 to 26 ml. were used to dissolve 250 mg. tryptophan). This solution was neutralized with normal HCl and diluted to 100 ml. with distilled water.

Technique.—To 0.3 ml. serum were added 0.9 ml. 0.9 per cent saline, 2.4 ml. 0.25 per cent tryptophan, and 3.6 ml. 60 per cent perchloric acid.

A control on the reagents was run simultaneously; this contained 1.2 ml. 0.9 per cent saline, 2.4 ml. 0.25 per cent tryptophan, and 3.6 ml. 60 per cent perchloric acid.

All tubes were gently shaken, covered with glass stoppers, and immersed simultaneously for exactly ten minutes in a vigorously boiling water bath. They were then removed simultaneously from the bath, cooled in water, and allowed to stand for forty minutes with occasional shaking to enable the precipitate to coagulate completely. The turbid pinkish-brown solutions were then filtered through small (70 mm.) filter papers (Whatman No. 42) until clear. If the solutions are not clear, the results will be too high. To prevent the precipitate from creeping over into the filtrate, the funnel is never filled more than half full. If creeping does occur, the solution must be refiltered.

The solutions were then read in the Coleman Junior spectrophotometer at 500 mμ against the reagent controls, which were set at zero.

Since the natural color of various sera differs considerably, controls on the sera were made by testing a solution of 0.3 ml. serum diluted with 6.9 ml. saline. The solutions were not heated, but simply read against a solution of saline.

The reading of the optical density scale of the Coleman instrument multiplied by 100 was recorded as Coleman units. The blank readings on the sera were subtracted from the readings on the unknown solutions and the differences recorded as the true reading.

From the Department of Internal Medicine, College of Medicine, University of Cincinnati, and the Gastric Research Laboratory of the Cincinnati General Hospital.

This thesis was submitted in partial fulfillment of the requirements for the degree of Master of Medical Science for graduate work in Internal Medicine to the Faculty of the Graduate School of Medicine, University of Pennsylvania, Philadelphia, Pa.

Received for publication, Dec. 5, 1949.

*Present address: 61 South Munn Ave., East Orange, N. J.

CLINICAL MATERIAL AND RESULTS

Sera from two groups of individuals were tested. An asymptomatic normal series of thirty individuals was chosen from doctors and nurses in the hospital whose ages ranged, with few exceptions, from 20 to 30 years. The other series of fifty-four cases was comprised wholly of cancer patients with varied symptoms referable to their new growth. These were all tested before the surgical removal of the lesions. Some of the patients with neoplasm had hypertension, inactive compensated cardiac disease, latent syphilis, or arteriosclerosis, but in none was there active inflammatory disease. There were two patients in the third decade, sixth in the 4th decade, eleven in the fifth decade, nine in the sixth decade, twelve in the seventh decade, thirteen in the eighth decade, and one in the ninth decade. In every instance the diagnosis of neoplasm was proved by biopsy or necropsy, which was performed in eleven patients of fourteen that died.

The various tumors were: cervical carcinoma, eight cases; gastric carcinoma, nine cases; colonic and rectal carcinoma, thirteen cases; esophageal carcinoma, two cases; carcinoma of the tongue, two cases; renal cell carcinoma, two cases; breast carcinoma, four cases; lung carcinoma, two cases; chronic myelogenous leucemia, two cases; brain tumor, one case; prostatic carcinoma, one case; multiple myeloma, one case; Hodgkin's sarcoma, one case; anal carcinoma, one case; and site undetermined, five cases. There were elevations above the normal range in at least one patient in every group mentioned with the exception of the case of Hodgkin's sarcoma which was at the upper limits of normal. Seven patients had localized lesions, twenty-eight had a primary tumor greater than 500 c.cm. in volume or metastases to regional nodes, and nineteen had a spread to distant organs. The size of the lesion was approximated in almost all instances by means of resected or necropsy specimens. In all likelihood the presence of distant metastases could not always be detected. The foregoing grouping, therefore, must be considered somewhat arbitrary.

The mean result in the cancer group was 24 units with a range from 9 to 45 units compared with a mean in the normal group of 14 units with a range from 8 to 20 units (Table I). A result of 20 units or greater, which represents a figure greater than twice the standard deviation above the normal mean, was considered to be a positive test. Thirty-nine of the fifty-four patients (72 per cent) had positive results on this basis. In the fifteen patients with results below 20 units, the lesions were localized in three instances, regional metastases or local extension were present in ten instances, and metastases to distant organs were present in only two instances. The degree of elevation of the test could not

TABLE I. SERUM ANALYSES

GROUP	NUMBER OF CASES	MEAN	RANGE	STANDARD DEVIATION
Normal	30	14 ± 0.5*	8-20	2.7
Cancer	54	24 ± 1.0*	9-45	7.1

*Results are in Coleman Units; the figure after ± represents the standard error. The standard error of the difference between the means is 1.1. Since the difference between the means, 10, is much more than twice 1.1, the results are highly significant.

be correlated with weight loss, duration of symptoms, degree of anemia, leucocytosis, age of the patient, or grade of malignancy of the tumor. In a general way, however, there was some correlation with the size of the malignant lesion or the presence of distant metastases. Thus in twenty-one patients with a volume of malignant tissue greater than 500 c.cm. or with multiple metastases to distant organs, only two had results below 20 units. Both of these were 19 units. The mean of this group was 28 compared with an over-all mean of 24 units in the patients with cancer.

COMMENTS

Since this test probably is a measure of tissue destruction, its nonspecific nature must be emphasized. The finding of positive tests in such conditions as chronic infections,[3] hyperthyroidism,[3] active cardiovascular disease,[3] cirrhosis,[3, 4] acute pancreatitis,[4] and even in a case of scurvy[4] is therefore not surprising. In a given case a positive test, therefore, merely represents a pathologic process that includes neoplastic disease. This cancer may still be at a "curable" stage since there were eighteen positive tests in the fifty-four patients with cancer in this series who had resectable lesions (no evidence of metastases beyond the site of removal) or lesions susceptible to radiation therapy (cervical carcinoma up to and including stage III). The total number of "curable" cases was twenty-eight, there being ten normal results and eighteen (64 per cent) positive results. This test, therefore, pointed toward a cancer being present in a majority of "curable" cases.

SUMMARY

1. In patients with malignant disease, in corroboration of the work of Seibert,[1] there was a significant elevation in a serum polysaccharide presumed to represent tissue destruction. Thirty-nine of fifty-four such patients had positive tests, i.e. a reading greater than twice the standard deviation above the normal mean.

2. Only two of the fifteen negative tests occurred in patients with distant metastases. The results in both these patients were at the upper limit of normal.

3. In a general way, only, could the test be correlated with the extent of the malignant process.

4. Of the twenty-eight cases amenable to surgical or radiation therapy, eighteen showed positive tests.

5. The finding of a positive test in conditions other than cancer is noted. In a given case, therefore, a positive result suggests an active pathologic process. Further definitive study is required to rule out a neoplastic process.

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STUDIES ON THE DEPRESSION OF BRAIN OXIDATIONS

II. Comparative Influence of Pentobarbital Upon Dog Cortex, Caudate Nucleus, and Hypothalamus

D. S. Wilkins, M.D.,* R. M. Featherstone, Ph.D., J. T. Schwidde, M.D., and M. Brotman, M.D.†
Iowa City, Iowa

THIS experiment compares the oxygen consumption of dog cortex, caudate nucleus of the basal ganglion, and hypothalamus with and without the influence of 0.04 per cent pentobarbital added in vitro. The project utilizes a method of dog brain biopsy and standard manometric technique with a multifactorial design, the results from which have been subjected to analysis of variance. The experiment follows a recent investigation by similar methods[1] which showed that a 0.04 per cent pentobarbital concentration in vitro resulted in a statistically significant, submaximal depression of dog cortex oxidations suitable for the comparison of different brain levels.

TECHNIQUE

By a modification of the method previously reported,[1] simultaneous biopsies of the cortex, caudate nuclei of the basal ganglia, and hypothalamus were secured from each of eight dogs. As before, the temporal muscles were partially excised and the calvarium was removed during sodium pentobarbital-nitrous oxide-oxygen anesthesia. The dura was incised and replaced without suturing, which procedure was necessary to permit access to the brain at subsequent biopsy without evoking pain and struggling. Approximately seven days later the incision was reopened during 1 per cent procaine anesthesia injected locally into the scalp. The dural flaps and granulations at the periphery were removed. The dog was sacrificed by the rapid removal of a coronal section, approximately 2.5 cm. in thickness, which contained the caudate nuclei of the basal ganglia, the hypothalamus, and cortex. The desired portions of the section were then excised within a few seconds and placed upon moistened, ice-cold filter paper.

The tissues were hashed for use according to standard manometric practices. About thirty-five minutes elapsed from the time of biopsy until oxygenation in the flasks was begun. Reaction vessels of about 4 c.c. volume with constants of about 0.4 were used.[20] Fluid volume in the calculation of the constants included 25 c.mm. in allowance for 25 mg. tissue samples. The flasks contained 0.5 ml. Krebs-Ringer phosphate buffer,[2] in which the calcium chloride content had been reduced to one-third of the recommended concentration to prevent the precipitation of calcium pentobarbiturate. The treatments comprised duplicates of (1) 0.04 and (2) 0.00 per cent sodium pentobarbital, as pentobarbital, added to the buffer solution before the flasks were filled. The pH was adjusted to 7.4 with 1 per cent HCl by means of a Coleman pH meter. Five hundredths milliliter of 10 per cent KOH and a filter paper

From the Divisions of Anesthesiology and Neurosurgery of the Department of Surgery and the Department of Pharmacology, College of Medicine, State University of Iowa.
Submitted by Dr. Wilkins to the Graduate College, State University of Iowa, in partial fulfillment of the requirements for the degree of Master of Science in Anesthesiology. Presented during the Interim Meeting of the American Society for Pharmacology and Experimental Therapeutics, Nov. 18, 1949, at Indianapolis, Ind.
Received for publication, Dec. 7, 1949.
*Captain, United States Army Medical Corps.
†Present address: Department of Anesthesia, Mount Sinai Hospital, Cleveland, Ohio.

were placed in the center well. One-tenth milliliter of glucose solution was added from the side arm at the end of ninety minutes to produce a final concentration of 0.025 per cent. Readings were taken for eight thirty-minute periods.

One of the duplicate samples was placed on each side of the water bath. Sample positions for a given side were randomized.

RESULTS

Fig. 1 illustrates the oxygen consumption of the control samples and of those to which 0.04 per cent pentobarbital had been added for cortex, caudate nucleus of the basal ganglion, and hypothalamus during each of the eight time periods. Each bar on the graph represents the cubic millimeters of oxygen

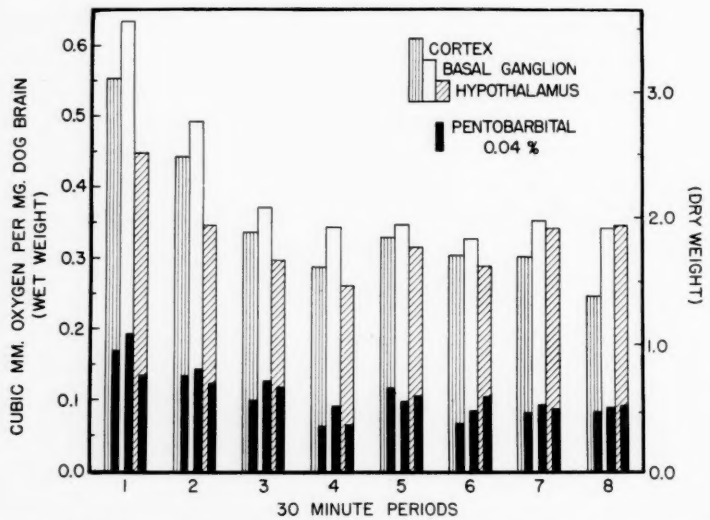

Fig. 1.—Graphic representation of oxygen consumption by brain levels. Basal ganglion refers to the caudate nucleus. Data illustrated are mean values of duplicate samples from eight dogs. Glucose in final concentration of 0.025 per cent was added at beginning of Period 4.

consumed per milligram of tissue during a thirty-minute period and is the mean of duplicate samples on eight dogs. Wet and dry weight scales are included. The dry weight scale was established on the basis of the wet to dry weight ratio, which had previously been found to equal 5.62 on separate aliquot samples of dog cortex. Dry weights have been computed for the illustrations only. The analysis of results has been calculated on a wet weight basis.

The mean oxygen consumption by the control samples of cortex during the first ninety minutes was 1.328 c.mm. per milligram of tissue, wet weight. In the previous investigation the mean value for five dogs in comparable terms was 1.117 c.mm., while Quastel and Wheatley[3] reported 0.780 cubic millimeters.

Figs. 2 and 3 are representative photomicrographs of sections from the caudate nucleus of the basal ganglion and hypothalamus, respectively. Cortical sections, not pictured, were similar to those shown in the previous publication. None of the sections evidenced inflammation, unusual cellular degeneration, or other abnormalities.

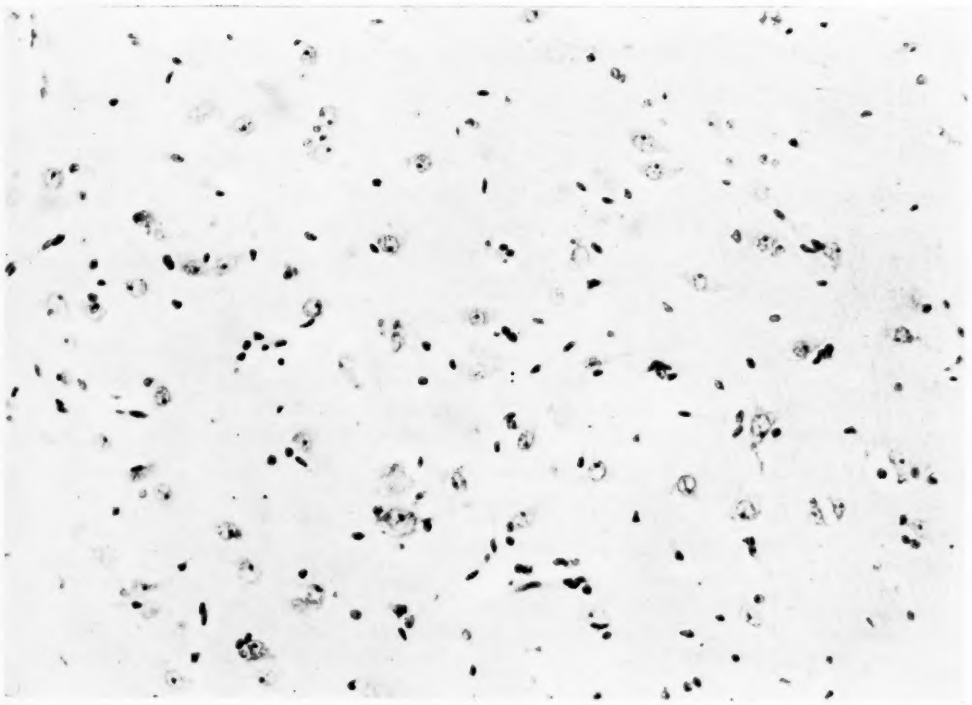

Fig. 2.—Photomicrograph of a section from a representative biopsy of dog caudate nucleus. (Magnification ×200.)

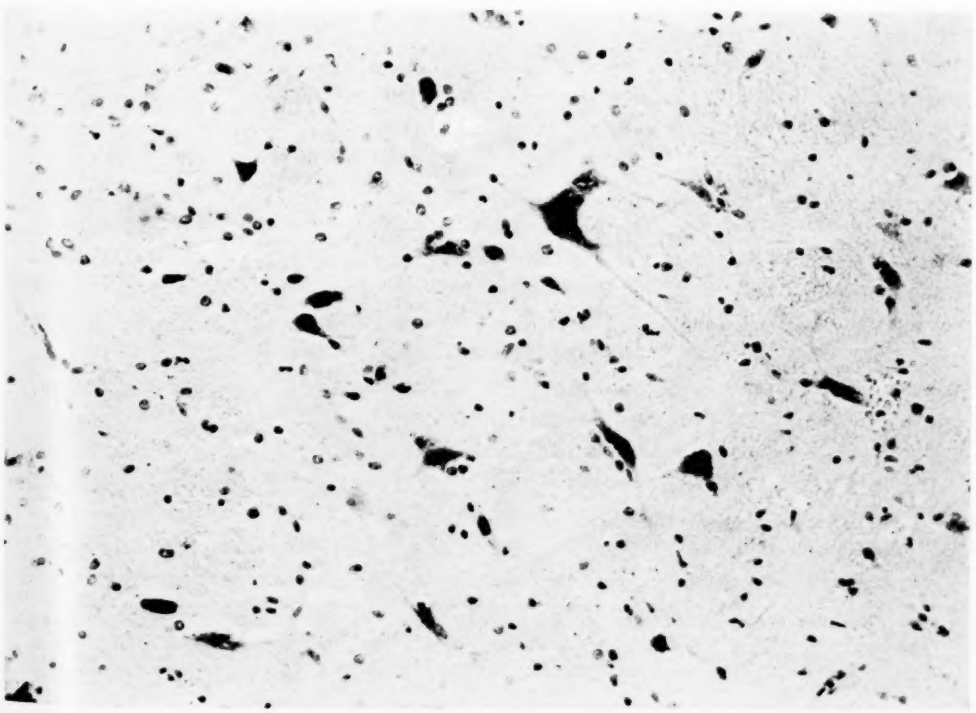

Fig. 3.—Photomicrograph of a section from a representative biopsy of dog hypothalamus. (Magnification ×200.)

INTERPRETATION OF RESULTS

The data from this experiment can be interpreted from three aspects: (1) by the analysis of the direct oxygen consumption data, (2) by the transformation prior to analysis of all data into terms of the per cent depression caused by pentobarbital, (3) by a logarithmic transformation of the data prior to analysis. It seems probable that each aspect should contain information pertinent to the study. The present discussion will deal only with the first aspect, and it is anticipated that the other two will be reported at a later date.

TABLE I. SUMMARY OF ANALYSIS OF VARIANCE FOR CONTROL AND PENTOBARBITAL TREATMENTS APPLIED TO CORTEX, CAUDATE NUCLEUS, AND HYPOTHALAMUS AND OBSERVED THROUGH PERIODS 1 TO 8 ON DOGS 1 TO 8

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARES	F	SIGNIFICANT F AT LEVEL OF 1% (**)	5% (*)
Dogs (D)	7	0.160929	0.022990	0.77	6.35	3.52
Levels (L)	2	0.240289	0.120145	4.02*	6.51	3.74
Caudate nucleus versus cortex†	1	0.132837	0.132837	4.45	8.86	4.60
Caudate nucleus versus hypothalamus†	1	0.217264	0.217264	7.27*	8.86	4.60
Cortex versus hypothalamus†	1	0.010332	0.010332	0.35	8.86	4.60
Error a						
D × L	14	0.418361	0.029882			
Treatments (T)	1	12.314028	12.314028	986.07**	8.02	4.32
T × L	2	0.113710	0.056855	4.55*	5.78	3.47
Error b	21	0.262264	0.012488			
T × D		0.115793				
T × D × L		0.146471				
Samples (S)	48	0.295902	0.006165			
Periods (P)	7	2.180221	0.311460	49.05**	2.71	2.04
Linear component	1	1.267706	1.267706	199.64**	6.73	3.87
Deviation from linear regression	6	0.912515	0.152085			
Second degree term	1	0.685731	0.685731	107.99**	6.73	3.87
Deviation from quadratic regression	5	0.226784	0.045357			
P × L	14	0.284888	0.020349	3.21**	2.15	1.73
Linear component	2	0.233307	0.116654	18.37**	4.68	3.03
Deviation from linear regression	12	0.051581	0.004298			
Second degree term	2	0.024018	0.012009	1.89	4.68	3.03
Deviation from quadratic regression	10	0.027563	0.002756			
P × T	7	0.542955	0.077565	12.22**	2.71	2.04
Linear component	1	0.254540	0.254540	40.08**	6.73	3.87
Deviation from linear regression	6	0.288415	0.048069			
Second degree term	1	0.207088	0.207088	32.61**	6.73	3.87
Deviation from quadratic regression	5	0.081327	0.016265			
P × T × L	14	0.119455	0.008532	1.34	2.15	1.73
Linear component	2	0.085253	0.042627	6.71**	4.68	3.03
Cortex†	1	0.184371	0.184371	29.03**	6.73	3.87
Caudate nucleus†	1	0.152512	0.152512	24.08**	6.73	3.87
Hypothalamus†	1	0.002910	0.002910	0.46	6.73	3.87
Deviation from linear regression	12	0.034202	0.002850			
Second degree term	2	0.014868	0.007434	1.17	4.68	3.03
Deviation from quadratic regression	10	0.019334	0.001933			
S × P for samples treated alike	336	0.920444	0.002739			
Error c	294	1.877145	0.006350			
Total	767	18.504245				

†Nonorthogonal comparisons.

As in the previous experiment, an analysis of variance[4] has been applied to the results.* The basic datum was the total of duplicate samples per treatment per level per period per dog, expressed in cubic millimeters of oxygen uptake per milligram of tissue, wet weight. Computation of the analysis was similar to that shown in the first report.

The analysis is summarized in Table I. The main sources of variation were dogs, (brain) levels, treatments, and periods. The mean square for each is the estimate of variance arising from its respective source. Errors a, b, and c, in general, are the appropriate estimates of experimental error for those sources of variation appearing above each in the summary table. A source of variation approaches significance as its mean square exceeds that for experimental error. Hence, the mean square for a source of variation is divided by the mean square for the proper error term, giving a ratio which is denoted as F. In Table I those F values which are significant at the 5 per cent level are labeled with one asterisk (*) and those significant at the 1 per cent level with two asterisks (**). In addition, the values above which F is significant at these levels are included.

It will be noted that the F for Dogs in Table I is not significant. F being less than unity indicates that the variation from dog to dog was less than experimental error.

The difference among all three brain levels, as indicated by F, was significant at the 5 per cent level. In separating the variation for levels into individual comparisons, it is found that basal ganglion (caudate nucleus) consumed significantly greater amounts of oxygen than did hypothalamus, that the oxidations of basal ganglion (caudate nucleus) were almost significantly greater than those of cortex, and that cortex and hypothalamus were nearly equal. These comparisons appear as Caudate nucleus versus hypothalamus, Caudate nucleus versus cortex, and Cortex versus hypothalamus in Table I. The differences are illustrated in Fig. 4, which shows the oxygen consumption in cubic millimeters per milligram of tissue totalled for all of the eight thirty-minute periods and expressed as the mean for eight dogs.

The difference between treatments, i.e., between the controls and those samples to which 0.04 per cent pentobarbital had been added, is represented by an extremely high F value (Treatments in Table I). It should be recalled, however, that the concentration of pentobarbital was chosen because it was known from earlier experimentation that it would provide such a difference.

The mean square for the Treatments × Levels interaction (T × L in Table I) is the estimate of the failure of the three brain levels to respond uniformly to the two treatments. The corresponding F is significant at the 5 per cent level, indicating with considerable assurance that the three brain levels did respond in unlike manners to the addition of pentobarbital. The significant T × L interaction reflects depressions by 0.04 per cent pentobarbital of cortex

*For their guidance in the analysis, the authors are grateful to G. F. Sprague, Senior Agronomist, United States Department of Agriculture, in charge of the cooperative corn breeding investigations at Iowa State College and to J. G. Darroch, Instructor and Research Associate, Statistical Laboratory, Iowa State College, Ames, Iowa. They acknowledge the helpful criticism of C. V. Winder, Department of Pharmacology, Parke, Davis & Company, Detroit, Mich.

and caudate nucleus oxidations, which were 98.1 and 112.6 per cent respectively, and of hypothalamus, which was depressed 89.3 per cent of the mean depression for all three levels.

Next in order in Table I is the mean square for Samples, which is an estimate of the variance between the duplicate samples. It is noted that the mean square for Samples is less than that for Error b. It may be concluded, therefore, that in this experiment duplicate samples measured in the 4.0 c.c. flasks and manometers provided sufficient accuracy.

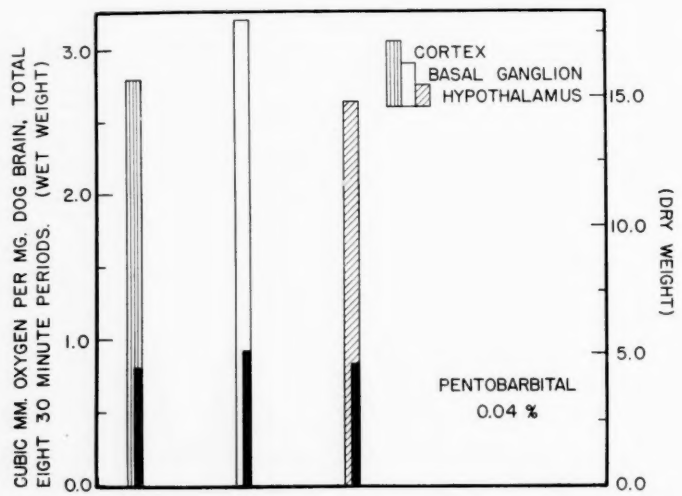

Fig. 4.—Same as Fig. 1, except that each bar is the total of the eight corresponding bars in Fig. 1. Basal ganglion refers to the caudate nucleus. Cortex was depressed by 98.1 per cent of the mean depression for the three brain levels, caudate nucleus by 112.6 per cent, and hypothalamus by 89.3 per cent. However, the addition of pentobarbital depressed cortex by 71.0, caudate nucleus by 71.2, and hypothalamus by 68.5 per cent of the respective uninhibited consumptions.

Continuing with Table I, it is seen that F for Periods is highly significant. Reference to Fig. 1 shows that this difference presumably resides in the decrease of oxidations with time. That this decrease with time is highly significant is demonstrated by F for Periods, Linear component[5] in Table I. There is also a pronounced tendency toward quadratic regression, as illustrated by F for Periods, Second degree term. It can be seen that the change in oxygen uptake with time was considerably less in those samples containing pentobarbital than in the controls. That this difference is highly significant is seen by F for P × T in Table I. Furthermore, the highly significant F for P × L indicates that the various brain levels responded differently by time periods.

The difference in response by the two treatments and by the three brain levels to time periods has been further studied by the ξ' method of fitting orthogonal polynomials.[5] The Linear component of the Periods × Levels interaction (P × L, Linear component in Table I) is seen to be significant beyond the 1 per cent level. Thus, if one were to construct according to time periods the mean of the two treatments for each brain level and draw for each level the straight line which best fitted the respective points, the slopes of the three

lines would differ with significance at the 1 per cent level. Since P × L, Second degree term (for quadratic regression) is nonsignificant, no information is gained for judging significance between curves, but the resulting quadratic curves fit the data with greater accuracy. The curves suggested are not illus-

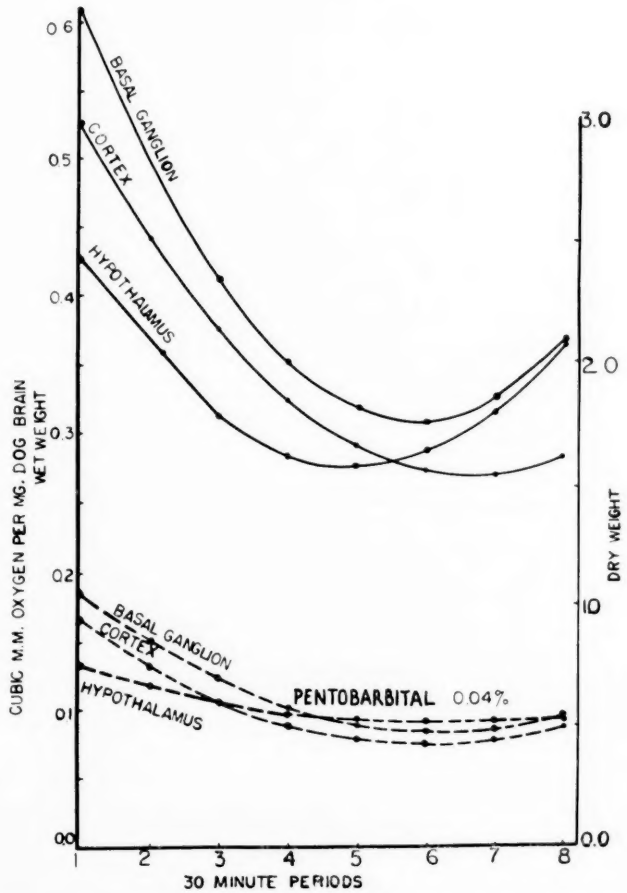

Fig. 5.—Graphic representation by quadratic regression of the mean data illustrated in Fig. 1. Glucose 0.025 per cent in final concentration added at beginning of Period 4. Basal ganglion refers to the caudate nucleus. Differences significant between linear slopes of the cortex and of the basal ganglion curves. Difference between linear slopes of the hypothalamus curves not significant. Relationships among slopes of control curves significantly different from those among slopes of pentobarbital curves. (See text.)

trated, but they are very similar in the relationships of the brain levels to the upper three curves in Fig. 5, and it is the type of variation among brain levels exemplified by these curves which is being measured. Similarly, P × T, Linear component, and P × T, Second degree term, in Table I measure the difference between the slope of the mean curve of the control curves and the mean curve of the pentobarbital curves in Fig. 5. It is noted that F for these terms is highly significant, indicating that the addition of pentobarbital retarded the decline in oxygen consumption with time.

Although Fig. 5 has been utilized in the discussion to illustrate P × T and P × L, it was drawn from the second order interaction Periods × Treatments × Levels (P × T × L in Table I). F is significant beyond the 1 per cent level for P × T × L, Linear component. This is interpreted to mean that the relationships of the three brain levels to each other manifest in the upper, or control, curves were altered by the addition of pentobarbital, i.e., the brain levels exhibit different relationships to each other in the lower, or pentobarbital, curves. Further examination reveals that the curve for cortex with pentobarbital is significantly different (beyond the 1 per cent level) from the control cortex curve, with respect to slope, not height, on the graph (F for P × T × L for Cortex, Linear component in Table I). The same is true for the pentobarbital and control curves for caudate nucleus of the basal ganglion (P × T × L for Caudate nucleus, Linear component). By contrast, however, the addition of pentobarbital to hypothalamus resulted in a curve essentially similar to its control (P × T × L for Hypothalamus, Linear component).

The regression coefficients determined by the ξ' method for the curves in Fig. 5 and for the other curves mentioned are listed in Table II.

TABLE II. COEFFICIENTS AND MEAN OBSERVATIONS FROM 8 DOGS, USED IN THE METHOD[5] FOR FITTING CURVES TO THREE BRAIN LEVELS AND TWO TREATMENTS ON THE BASIS OF TIME PERIODS 1 TO 8

INTERACTION	CURVE	MEAN OBSERVATION FOR CURVE	REGRESSION* COEFFICIENTS	
		MM. O_2/MG. TISSUE FOR ONE SAMPLE	LINEAR	QUADRATIC (2ND DEGREE)
		A'_0	A'_1	A'_2†
P × L‡	Cortex	0.2256	−0.0113	0.0057
	Caudate nucleus	0.2578	−0.0118	0.0082
	Hypothalamus	0.2166	−0.0035	0.0056
P × T‡	Pentobarbital	0.1067	−0.0049	0.0029
	Control	0.3600	−0.0128	0.0101
P × T × L§	Control:			
	Cortex	0.3498	−0.0171	0.0080
	Caudate nucleus	0.4004	−0.0171	0.0127
	Hypothalamus	0.3297	−0.0042	0.0096
	Pentobarbital:			
	Cortex	0.1014	−0.0054	0.0035
	Caudate nucleus	0.1152	−0.0065	0.0038
	Hypothalamus	0.1035	−0.0028	0.0016

*As computed for use in this method for fitting orthogonal polynomials. For example, in this instance $A'_1 = b/2$, where b is the linear regression coefficient in the equation $Y = \bar{y} + b(x-\bar{x})$.[5]

†Designation used by Anderson and Houseman.[5]

‡Curves discussed in the text.

§Curves appearing in Fig. 5.

Barring artifact, it is believed that the experiment amply demonstrates differences, as measured by manometric techniques and with the addition of glucose at ninety minutes, among the oxygen utilizations of cortex, caudate nucleus, and hypothalamus. They consume different quantities of oxygen in the uninhibited state. They are depressed unequally by 0.04 per cent pentobarbital added in vitro, as computed directly from the oxygen consumption data. They vary in the character of their respective depressions. The asser-

tion cannot be made that the differences are physiologically important in terms of function on the basis of their statistical significance in this experiment. Such a conclusion must await further investigation. However, the variation among the three brain levels deserves an effort to reach a satisfactory explanation.

As has been stated, the data are based upon 25 mg. (wet weight) samples. The cell types, sizes, and frequencies are not uniform among the brain levels studied. This variation in cell population may have influenced the results cited, and this factor is being subjected to further study.

No references to the particular comparisons recorded in this investigation have been found in the literature. A number of authors, however, have made contributions pertinent to such a study. Worthy of mention are those relating to the disagreement as to the merits of the brain slice and hash techniques. Dixon and Meyer[6] have reported the oxygen uptake in microliters per gram (wet weight) by ox brain for a two-hour period as being 1,600 for minced tissue and 4,000 for slices. Quastel and Wheatley[3] observed that chopped and sliced brain yielded comparable uptakes, and Quastel[7] quotes Ashford and Holmes as having obtained a 16 per cent reduction of the slice values by chopping. In the same review of respiration in the central nervous system Quastel states that Jowett has shown that the respiration of sliced brain is more sensitive to a narcotic such as ethyl urethane than was the respiration of the minced brain used by Bulow and Holmes. Jowett and Quastel[8] confirmed with brain slices an earlier research of Quastel and Wheatley,[9] which was conducted with chopped brain, regarding the inhibition of the oxidation of various substrates by barbiturates.

The influence of narcotics upon brains of several animals, as studied by manometric techniques, has been reported extensively.[9-14] Quastel and Wheatley[9] have published curves showing a diminished slope of oxidations with time upon the addition of barbiturate, which appear similar to the results of this investigation. Quastel[7] has reviewed data tending to show that in vitro depressions of brain oxidations can be obtained by concentrations of a magnitude similar to those produced by narcotizing doses in vivo.

Comparisons of several brain levels by a variety of methods with and without depressants have been made by various authors[6, 7, 15-18] previous to this study. Of these, one is pertinent to the current investigation. Dixon and Meyer[6] compared cortex, caudate nucleus, and thalamus-hypothalamus of ox brain without the influence of depressant drugs. By inspection their data apparently show the highest oxygen uptake for caudate nucleus, the next for cortex, and the least for thalamus-hypothalamus. In addition, their one-, two-, and three-hour observations seem to indicate the least slope for the thalamus-hypothalamus. An impression is gained that their results illustrate the same tendencies which have been quantified by the analysis of variance, graphs, curves, and regression coefficients in the present research.

SUMMARY

This experiment compares the oxygen consumption of dog cortex, caudate nucleus of the basal ganglion, and hypothalamus with and without the influence

of 0.04 per cent pentobarbital, which concentration had been shown earlier to produce a statistically significant, submaximal depression of dog cortex oxidations. A method of dog brain biopsy was combined with multifactorial design and analysis of variance for a procedure utilizing standard manometric techniques with 4 c.c. reaction vessels.

Based upon the statistical analysis, as applied directly to the oxygen consumption data, the following conclusions are drawn.

1. The variance among the dogs used was not significant.

2. The total oxygen consumption per unit wet weight by the caudate nucleus of the basal ganglion was greater than that by hypothalamus, with significance approaching the 1 per cent level.

3. The total oxygen consumption by the caudate nucleus of the basal ganglion was not shown to be different from that of cortex, although the greater value for the former approached the 5 per cent level of significance.

4. The total oxygen consumptions by cortex and hypothalamus were almost identical.

5. Pentobarbital, 0.04 per cent, depressed cortex by 98.1 per cent of the mean depression, caudate nucleus by 112.6 per cent, and hypothalamus by 89.3 per cent. This difference in the response of the three brain levels to pentobarbital was significant at the 5 per cent level. In the same order the levels were depressed by 71.0, 71.2, and 68.5 per cent of their respective uninhibited consumptions.

6. The three levels and the two treatments responded differently according to time.

7. The relationships to each other of the oxidations by brain levels as plotted against time was altered by the addition of 0.04 per cent pentobarbital, with significance at the 1 per cent level. This is a reflection of the fact that the curves of cortex and caudate nucleus oxidations plotted against time are each altered by the addition of 0.04 per cent pentobarbital with significance at the 1 per cent level, but that of hypothalamus is not so changed.

8. The 4 c.c. flasks and corresponding manometers provided sufficient accuracy with duplicate samples.

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STAINING OF ERYTHROCYTES OF DIABETIC AND NONDIABETIC PATIENTS: THE UNRELIABILITY OF THE BREMER TEST

MILTON B. HANDELSMAN, M.D., LEON M. LEVITT, M.D., AND
LEONARD RUBIN, M.A.
BROOKLYN, N. Y.

IN 1894, Bremer[1] described a method of diagnosing diabetes mellitus by the differential staining of blood smears of patients with glycosuria. This method consisted of empirically mixing solutions of eosin and methylene blue, so that eventually, by trial and error, a proper mixture was found in which the erythrocytes obtained from diabetic persons stained green and those from nondiabetic persons stained pink. In 1896 Bremer[2] further simplified the test, leading to its use in many continental clinics such as those of Lépine, P. Marie, and Von Norden. Essentially the newer test consisted of fixing blood smears in an oven at 135° C. for eight to ten minutes and then staining the fixed smear in a 1 per cent aqueous solution of Congo red or methylene blue. The erythrocytes of diabetic patients were reported to be stained by these dyes and those of nondiabetic persons were unstained. Biebrich scarlet, on the other hand, reacted in opposite manner.

A vast amount of investigation was carried on to study the nature of the Bremer test and three theories were proposed. The originator believed that the differences in staining were due to the affinity for the dyes of some unknown substance occurring in the diabetic erythrocyte. Glucose was not this unknown substance, since he could not get positive tests by staining blood smears of animals which had been given glucose parenterally. Since blood smears of nondiabetic subjects, when floated on diabetic urines, gave positive tests, he postulated that the unknown substance was excreted in the urine.

Marie and Le Goff,[3] repeating Bremer's observations in diabetic patients, believed that the unusual staining reaction indicated a degeneration of the constituents of the erythrocytes, probably a change in the nature of the hemoglobin in diabetic cells. Hartwig[4] subscribed to this view and pointed out that positive tests could be obtained also with the blood of patients suffering from leucemia, Graves's disease, and multiple neuritis.

Lépine and Lyonnet,[5] corroborating Bremer's work, believed that the positive staining was due to a decreased alkalinity or an increased acidity in diabetic erythrocytes, allowing them to accept basic aniline dyes. Loew, Eichner and Volkel, and Schneider, quoted by Hartwig,[4] subscribed to this interpretation of the test.

Although Bremer mentioned in various articles that his correspondence included reports from physicians who could not confirm his test, only one such negative report was found by us in the literature. The Bremer test was

From the Diabetic Clinic of the Long Island College Hospital and the Department of Medicine, Long Island College of Medicine.

This investigation was supported by grants from Burroughs Wellcome & Co., Inc., and the Julia and Samuel Bernstein Foundation.

Received for publication, Dec. 10, 1949.

gradually replaced by the simpler Williamson test, which was not a staining reaction but an actual test tube reduction of methylene blue by whole blood. The advent of micromethods for blood sugar estimation replaced both. In view of the clinical implications of the Bremer test, this work was undertaken to study its validity.

METHODS

The "Isoelectric Staining Zone" of Normal and Diabetic Erythrocytes.—Bacteriologists have determined an "isoelectric zone" of bacterial proteins by staining methods. This zone is defined[6] as that pH at which acid dyes are rejected and staining by alkaline dyes occurs, or vice versa. Tolstouhov[7] determined this zone in erythrocytes and showed that although it varied depending on the fixation employed, it remained fairly constant when the same fixing agent was used.

In order to determine whether an abnormal constituent or a qualitative change in the protein constituents existed in the erythrocytes of diabetes, Tolstouhov's method of staining was applied to methanol-fixed smears obtained from uncontrolled diabetic subjects and nondiabetic persons. Alcoholic solutions of eosin-methylene blue were adjusted to various pH values by adding N/100 hydrochloric acid or sodium hydroxide according to the tables given by Tolstouhov. Staining of smears was done in Coplin jars. Gross macroscopic observation gave good indication of the color accepted by the erythrocytes. Microscopic examination was more confusing, inasmuch as the thickness or thinness of the smear causes a large variation in the staining of the erythrocytes. However, a close correlation between macroscopic and microscopic recordings was obtained if the latter was done on the thin part where the red corpuscles were not contiguous. The staining intensity was graded according to an arbitrary scale, a task which was not difficult after some experience had been gained.

RESULTS

Table I summarizes the results found in controls and diabetics. A change in staining affinity occurred between the pH of 4.8 and 4.95 in most patients, the red color of eosin appearing in the erythrocytes at the lower pH and the methylene blue staining the erythrocytes greenish-blue at the higher pH. Some erythrocytes stained bluish in the methylene blue and some had a greenish shade; no cause for this difference was found. It was difficult to determine a sharp change when the pH changes were less than 0.15. As Table I demonstrates, no differences were found in the staining reactions of the methanol-fixed erythrocytes of diabetic and nondiabetic persons.

It must be emphasized that these pH values have no absolute significance despite the consistency found when the same fixative is employed. For instance, substitution of acetate or phosphate buffers for the hydrochloric acid and sodium hydroxide used by Tolstouhov to vary the pH of the dye does not give comparable staining results at the same pH levels. However, the consistency found from day to day when the method is kept constant allows a valid comparison of various patients' bloods taken at different times.

The Staining of Heat-Fixed Erythrocytes of Normal and of Diabetic Patients With Aqueous Methylene Blue Solution.—After much experimentation, Bremer[2] reported that heat fixation of erythrocytes gave more constant results with his test. Schneider[4] also reported that only heat fixation was found to give consistent results at the Heidelberg Clinic. Thus, the Bremer test was simplified in 1899 so that finally it depended on the difference that heat-fixed

TABLE I. STAINING AFFINITY OF METHANOL-FIXED ERYTHROCYTES FOR ALCOHOLIC SOLUTIONS OF EOSIN-METHYLENE BLUE AT VARYING pH LEVELS (TOLSTOUHOV METHOD)

The staining is recorded as red (R), green (G), or blue (B). The varying intensities of staining were arbitrarily graded by fractions denoting an approximate decrease of staining intensity

	pH						
	4.15	4.5	4.8	4.95	5.1	5.25	REMARKS
Nondiabetics							
Student	R	¾ R	½ R	½ G	G	B	
Student	R	¼ R	¼ R	¼ G	G	B	
Physician	R	½ R	½ G	¾ G	G	B.....	More basophilic
Physician	R	½ R	?G R	½ G	G	B....?	More basophilic
Janitor	R	R	½ R	¼ G	½ G	B	
Educator	R	¾ R	¼ R	¼ B	B	B	
Nurse	R	R	¼ R	¼ B	½ B	B	
Educator	R	R	½ R	¼ B	¼ B	B	
Physician	–	R	¼ R	¼ R	¼ B	B.....	More acidophilic
Controlled diabetics (without glycosuria)							
A. S.	R	½ R	¼ R	¼ B	¾ B	–	
E. Q.	R	R	½ R	¼ G	¾ G	B	
M. T.	–	R	R	¼ B	½ B	¾ B	
J. T.	R	R	R	¼ B	½ B	–	
R. V.	R	¼ R	¼ B	¼ B	–	–.....	More basophilic
R. S.	R	R	¼ R	¼ R	¼ B	–.....	More acidophilic
Uncontrolled diabetics							
J. S. H.	R	½ R	¼ R	¼ B	¼ B	B	
M. M.	R	R	½ R	½ G	¼ G	B	
C. G.	R	½ R	½ B	½ B	B	B.....	More basophilic
D. A.	–	R	¼ R	¼ B	?	–	
S. P.	–	R	¼ R	B	B	–	
H. M.	–	R	¼ R	?	B	–?....	More acidophilic
Other diseases							
Cirrhosis (liver)	–	R	¼ B	½ B	B	–.....	More basophilic
Rheumatic H. D.	–	½ R	¼ R	¼ B	¼ B	–	
General arterio.	–	R	¼ R	¼ B	¼ B	–	
Rheumatic fever	–	R	½ B	B	B	–.....	More basophilic

erythrocytes of diabetic persons were stained deep blue by a 1 per cent aqueous solution of methylene blue while the red cells of nondiabetic persons, under the same staining conditions, stained light green.

That fixation of erythrocytes by heat might lead to such a differentiation seems plausible since it is known[8] that glucose in the presence of heat can denature proteins by affecting the NH2 radical of the protein. Furthermore, Le Goff[9] has shown that hemoglobin heated with glucose becomes more acidic and thus stainable by methylene blue. Drabkin[10] pointed out that despite the apparent differences in the amount of glucose and hemoglobin in erythrocytes, when these are expressed in molecular concentrations per cell, they actually have stoichiometric significance ($2.6 \times 10\text{-}16$ moles glucose and $3.1 \times 10\text{-}16$ moles Hgb. per cell).

A technician in our laboratory followed Bremer's method very closely. Smears were pulled on clean, new, glass slides in the usual manner. The slides were fixed in a Fisher Junior oven for fifteen minutes with the oven thermometer at 125° C. and then stained for three minutes in a Coplin jar containing aqueous methylene blue solution. Most of the smears obtained from diabetics

were stained blue and most from nondiabetics were either light green or did not accept the dye. It was soon evident that this was accidental, since no consistency in results could be obtained. A smear test that was positive was often negative on repetition, and so forth. It was obvious that conditions had to be more carefully controlled than was described by Bremer.

Further studies revealed that the results of staining were dependent on at least four factors: (1) the heat of fixation, (2) the percentage of dye concentration, (3) the pH of the staining solution, and (4) the pH of the wash solution. Each of these factors was studied separately and in combinations.

Regarding the *heat of fixation*, it was found that the temperature in the Fisher Junior oven varied from approximately 170° C. on the lowest shelf to approximately 135° C. on the highest shelf when the thermostat control was adjusted so that a routine reading of the oven thermometer was 125° C. All smears fixed on the lowest shelf gave positive Bremer tests; those fixed on the upper shelf gave inconstant results.

It was also found that the *concentration of the dye* played a role in the staining. Medicinal methylene blue was employed; this was 85 per cent pure dye. No staining of the heat-fixed smears took place with 1 per cent solution as employed by Bremer. It was found that a 1 per cent solution of the dye employed had a pH of 3.8 (the pH of the distilled water solvent was 5.9). Further dilution of the dye led to an increase in the pH of the solution and led to better staining. Best staining of properly fixed smears was obtained with 0.15 per cent solution of methylene blue.

Table II shows a typical experiment performed with smears obtained from a patient with severe diabetes who had marked hyperglycemia after insulin had been discontinued for forty-eight hours and also from a normal nondiabetic subject. The three factors just discussed were controlled for each set of smears. At the lower temperature of fixation, 130° C. (actual temperature on the fixing tray in the oven), neither diabetic or nondiabetic bloods accepted the dye at any dilution or any pH. On the other hand, fixation at 170° C. led

TABLE II. THE EFFECT OF HEAT OF FIXATION, DYE CONCENTRATION, AND PH OF DYE SOLUTION ON THE STAINING OF ERYTHROCYTES BY AQUEOUS SOLUTIONS OF METHYLENE BLUE

% METH. BLUE SOL. IN DISTILLED WATER	PH OF SOL. AT ONSET	PH AFTER STAINING 10 SMEARS	FIXATION AT 130° C.		FIXATION AT 170° C.	
			DIABETIC	CONTROL	DIABETIC	CONTROL
0.06	4.8	6.6	0000000000,	00000	31	333,10
0.25	4.3	5.7	000000	00,0	3333	11031,13
0.50	4.0	4.7	000	00000,0	1313111	13,30
1.0	3.8	4.2	00	00	0000,0000	111111,11

The staining was done in Coplin jars for three minutes and after ten smears were stained the solutions in the jars were changed. The results are recorded in the order of staining continuously from left to right with a comma denoting a change of solution after the tenth slide. Each recorded pH was measured by glass electrodes. The smears were washed with a N/20 acetate buffer (pH 5.0).

The code for recording the staining of each individual slide is as follows:

0, No staining of the smear.
1, A light green staining.
2, A darker green staining.
3, A blue staining.

to staining of most smears. This staining was very poor with a high dye concentration (1 per cent), but was appreciably better with a more dilute solution of the dye.

This latter effect may be partly due to changes in pH of the dye solutions, since more dilute aqueous solutions of the medicinal methylene blue were relatively less acid (pH 4.8) than concentrated solutions (pH 3.8). However, the pH of the dye solution cannot be the only cause for the ability to retain the stain, since after using the dye in a Coplin jar to stain ten smears consecutively three minutes each, there was an increase in the pH of the dye solution without any perceptible enhancement of red cell staining. Obviously another unknown factor causing this must exist, but under the controlled conditions in this and many other experiments no differences in staining reaction between diabetic and nondiabetic blood smears were found.

In order to further explore a possible staining difference between diabetic and nondiabetic erythrocytes, the fourth factor mentioned, namely the effect of changing *the pH of the wash solution*, was investigated. Table III gives the results of two typical experiments. In these experiments, smears were drawn so that diabetic blood occupied one half of a slide and nondiabetic blood the other half. Experiment 21 was performed with the blood of a newly diagnosed,

TABLE III. THE EFFECT OF HEAT OF FIXATION AND PH OF THE WASH SOLUTION ON THE STAINING OF ERYTHROCYTES BY 0.15 PER CENT AQUEOUS METHYLENE BLUE

The smears were drawn so that diabetic blood and normal blood were on the same slides. The washing solutions were buffered with N/20 acetate buffers. The recording of the staining results on each slide is the same as described in Table II. Two sets of experiments are given to show that even under controlled conditions staining intensity was different at different times

TEMPERATURE OF FIXATION (15 MIN.)	EXPERIMENT	WASHED AT PH 4.4		WASHED AT PH 5.0		WASHED AT PH 6.2	
		DIABETIC	CONTROL	DIABETIC	CONTROL	DIABETIC	CONTROL
135° C.	21	00000	00000	00000	00000	31111	30030
	22	00000	00000	00000	00000	11110	10110
150° C.	21	00000	00000	00111	00101	33031	33131
	22	00000	00000	00010	01000	11011	10010
170° C.	21	00000	00000	01111	01101	31331	30330
	22	00000	00000	01311	01301	31011	30100

untreated diabetic patient and compared on the same slide with the blood of a healthy medical student. Experiment 22 included the blood of an uncontrolled diabetic patient who had discontinued insulin for seven days as compared with the blood of another healthy medical student. In these experiments the staining solution was constantly taken from a stock solution of 0.15 per cent aqueous, unbuffered methylene blue and changed after staining five slides in a Coplin jar. The experiments were set up so that nine sets of five smears each were fixed at three different temperature levels. After staining, the sets were washed at three different pH levels. Washing the slides at a pH of 4.4 led to complete destaining. At a pH of 5.0, only those slides fixed at higher temperatures were slightly stained. Those washed at a pH of 6.2 were slightly better though erratically stained. The results given in Table III as well as in

other experiments not only showed no significant differences between diabetics and controls but also showed varied staining qualities of erythrocytes from the same person, even when conditions were carefully controlled. Since at the time the Bremer test was in vogue these factors were unknown and uncontrolled, it would seem that the old published results are unreliable.

CONCLUSIONS

1. The "isoelectric staining zone" of methanol-fixed erythrocytes is fairly constant between pH 4.8 and 4.95. There is no difference in this staining zone in erythrocytes of normal human beings and those of uncontrolled diabetic subjects.

2. Staining of heat-fixed erythrocytes in aqueous methylene blue as in the Bremer test for diabetes requires careful control of conditions.

3. The results obtained in such staining varies according to the temperature of fixation; the higher temperatures of fixation (above 150° C.) lead to good staining.

4. Unbuffered aqueous solutions of methylene blue are acidic; the acidity diminishes with dilution so that only very dilute solutions (0.15 per cent) lead to fairly good staining of blood smears.

5. Increased pH of the wash solutions allows better retention of the methylene blue.

6. With accurate control of these factors in staining the erythrocytes of uncontrolled diabetic subjects and nondiabetic persons, no differences in staining were found between the two.

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A STUDY OF THE EFFECT OF NEOMYCIN AND OTHER ANTIBIOTICS ON BACTERIA, VIRUSES, AND PROTOZOA

OSCAR FELSENFELD, M.D., ITALO F. VOLINI, M.D., AND SACHIKO J. ISHIHARA, B.S., CHICAGO, ILL., MARVIN C. BACHMAN, M.S., TERRE HAUTE, IND., AND VIOLA MAE YOUNG, M.S., CHICAGO, ILL.

NEOMYCIN, an antibiotic recently isolated and described by Waksman and co-workers,[1, 2] was found to be active against selected strains of *Mycobacterium tuberculosis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, and *Staphylococcus aureus* in vitro; against *Salmonella schottmülleri* and *Salmonella pullorum* in the developing chick embryo; and against *Staph. aureus*, *S. schottmülleri* and *Salmonella typhosa* in mice. The inhibition of *Euglena gracilis* by neomycin was observed by Dr. Ralph E. Bennett of the Commercial Solvents Corporation[3] who suggested that the drug was likely to inhibit other protozoa. According to Waksman and Lechevalier[1] the toxicity of this new antibiotic is low, and resistant bacterial forms did not develop under the influence of neomycin. Thus it seemed important to test the effect of this drug on a larger series of microorganisms and to compare its action with that of other antibiotics.

MATERIALS AND MEDIA

Neomycin, polymyxin D, enniatin III, bacitracin, and penicillin G were received from the production and research departments of the Commercial Solvents Corporation, Terre Haute, Ind. The activity of neomycin was 0.2 unit per microgram and that of polymyxin D, 0.6 unit per microgram.

Antibiotics designated as enniatin A, B, or I, II, etc., are practically insoluble, in water, and have been prepared from Fusaria, especially *Fusarium oxysporum* Schlecht.[4] The material used by us was a Commercial Solvents Corp. product, marked enniatin III. Because of the lack of an established assay method, the activity of this antibiotic was measured in micrograms per milliliter medium or per kilogram animal weight.

Streptomycin was received through the courtesy of The Upjohn Company, Kalamazoo, Mich.; aureomycin from the Lederle Laboratories, Pearl River, N. Y.; and Chloromycetin from Parke, Davis & Co., Detroit, Mich.

Most of the test organisms were recently isolated strains from patients of the Cook County Hospital in Chicago. Pathogens locally not available were received through the kindness of Dr. G. Pandit of the Calcutta School of Tropical Medicine, Dr. H. Seneca of Columbia University, Dr. V. Zichis of the Markham Laboratories in Chicago, and Dr. F. I. Huddleson of Michigan State College. Listeriae and Mycobacterium spp. were procured from the American Type Culture Collection.

Bacteria not requiring special nutrient media were grown and tested in Penassay broth, using the routine eighteen-hour turbidimetric method. Mycobacteria were cultured in Dubos fluid; Brucellae and Listerellae in Trypticase soy broth; Clostridia in semifluid Trypticase broth; Treponemataceae in Savino's fluid; intestinal protozoa in Dobell and Laidlaw's medium; and blood flagellates on N.N.N. agar.

Five to 10 M.L.D. of the tested microorganisms were used to infect experimental animals. At least six control and six test animals were used for each dilution of the examined antibiotic. For the evaluation of the drugs on enteric organisms two methods

From the Department of Bacteriology of the Hektoen Institute for Medical Research of Cook County Hospital, Chicago, Ill., and from the Division of Pharmaceutical Research of the Commercial Solvents Corporation, Terre Haute, Ind.

Received for publication, Nov. 21, 1949.

were used. In one the animals received the antibiotic two days before, on the day of, and two days after infection. In the second test the animals received the drug only once, i.e., simultaneously with the infective dose.

Myco. tuberculosis was tested in mice, using the established technique of intravenous infection of Raleigh and Youmans,[5] while action on *Endamoeba histolytica* was examined with the aid of Stewart and Jones's[6] method consisting of operative implantation of the amebas into the cecum.

The influence of drugs on vaccinia and herpes simplex virus was tested in rabbits by corneal scarification and subsequent local treatment of the right eye after 4, 24, and 28; 8, 24, and 48; and 12, 24, and 48 hours, while the left eye served as an untreated control. These viruses, as well as the Lansing strain of poliomyelitis, eastern and western equine encephalomyelitis, were tested in mice using the intracranial route of infection, while the mouse-adapted strain of influenza virus was introduced by the intranasal route. Guinea pigs were employed to test Rickettsiae of rickettsialpox and the virus of lymphocytic choriomeningitis.

These animals received the drugs for two days before and the day of the infection. After this, daily injections of the respective antibiotic were given until at least 80 per cent of the control animals died or, as in rickettsialpox and *Trypanosoma brucei* infections, until the controls ceased to manifest symptoms or harbor organisms.

Decreasing doses beginning with 100 U. or 100 micrograms per milliliter fluid medium were used in test tube experiments. Animal tests were first set up with one-half of the LD_0 of the respective antibiotic and, if this dose was found effective, decreasing amounts of the drug were injected. The one-dose LD_0 for mice and guinea pigs per kilogram weight for intra peritoneal administration was found to be 300,000 U. of penicillin, 100 mg. of streptomycin, 50 mg. of Chloromycetin, 30 mg. of aureomycin, 20 mg. of polymyxin D, 5,000 U. of bacitracin, 20 mg. of enniatin, and 200 mg. of neomycin. Thus the neomycin used by us had only one-half of the toxicity of streptomycin. It has to be added that enniatin caused local irritation. For this reason it was necessary to give the enniatin intramuscularly and for comparative purposes the other antibiotics were likewise administered by this route. Bacitracin and polymyxin D did not produce microscopically or histologically apparent kidney damage when given in doses equal to their respective LD_0 or less. Bacitracin caused cloudy swelling of the kidneys in about 10 per cent of the mice when injected in doses of 10,000 U. per kilogram or more but did not produce such an effect when administered orally in doses smaller than 50,000 U. per kilogram.

The amount of neomycin used in these experiments was expressed in weight and not in antibiotic units in order to compare it with other antibiotics from Actinomycetales. One microgram of the drug was found to be equivalent to 0.2 unit. Expressed in units, the activity of neomycin would appear much higher than in the present statistics. It was felt, however, that such a method of recording could be misleading and was therefore not used.

RESULTS

Tables I to IV show most of the results of the experiments, giving the number of units (penicillin and bacitracin) or micrograms per milliliter of medium or kilogram animal weight necessary to inhibit the growth in vitro or to prevent the formation of microscopic or histologic lesions in at least 90 per cent of the animals.

Gram-positive organisms were inhibited with smaller doses of neomycin than of streptomycin. The efficacy of neomycin was, however, much lower than that of other drugs. When the therapeutic index (LD_0 : active dose) is calculated, bacitracin proves to be an excellent agent against both gram-positive cocci and rods.

TABLE I. COMPARISON OF THE ACTION OF ANTIBIOTICS ON GRAM-POSITIVE ORGANISMS IN VITRO*

ORGANISM	NUMBER OF STRAINS TESTED	PENICILLIN			STREPTOMYCIN			CHLOROMYCETIN			AUREOMYCIN			POLYMYXIN D			BACITRACIN			NEOMYCIN		
		MA	MI	A	MA	MI	A	MA	MI	A	MA	MI	A	MA	MI	A	MA	MI	A	MA	MI	A
Staph. aureus	20	M	.03	4.6	M	1.25	42.2	M	.31	9.4	25	.01	1.1	M	2.5	8.7	12.5	.01	6.4	M	.03	21.1
α-Streptococci	30	M	.01	.02	M	.61	7.9	M	1.25	2.7	12.5	.01	.9	M	.03	1.3	12.5	.01	4.2	M	.03	5.5
β-Streptococci	50	M	.01	.03	M	.31	5.1	M	.62	3.4	2.5	.01	1.1	M	.06	3.3	25	.01	3.7	M	.03	3.2
B. anthracis	1		.06			M			25			12.5			1.25			1.0			2.0	
Clostridia	10	M	.06	7.4	M		M	M		M	M		M	M		M	12.5	.62	3.2	M		M
Listeriae	3	M	M	M	M	1	70.3	25	.03	8.8	1.25	.03	.5	.07	.03	.05	6.25	2.5	5.8	1.25	.03	.5

M, More than 100 micrograms or units.
Ma, Maximum; Mi, minimum; A, average.
*Penicillin and bacitracin end points expressed in units per milliliter, all others in micrograms per milliliter.

TABLE II. ACTION OF ANTIBIOTICS ON GRAM-NEGATIVE RODS IN VITRO*

ORGANISM	NUMBER OF STRAINS TESTED	STREPTOMYCIN			CHLOROMYCETIN			AUREOMYCIN			POLYMYXIN D			NEOMYCIN		
		MA	MI	A	MA	MI	A	MA	MI	A	MA	MI	A	MA	MI	A
S. typhosa†	10	M	M	M	12.5	.62	4.3	12.5	.31	3.1	1.25	.15	.7	5	.62	2.4
S. typhosa‡	10	50	4	44.3	12.5	.62	4.1	12.5	.15	2.2	2.5	.07	.5	5	.31	1.9
S. paratyphi A, B, and C	6	M	2	13	25	.31	2.2	12.5	.31	2.7	5	.07	.4	20	.31	.8
Other Salmonellae	30	M	1	17.3	50	.31	5.2	25	.15	5.8	25	.07	5.2	25	.62	6.2
Shigellae	42	100	.62	5.2	25	.31	2.2	12.5	.15	2.1	5	.03	1.2	20	.31	5.1
Esch. coli	20	M	1.25	5.8	50	1.25	5.3	50	.31	5.2	12.5	.07	1.17	20	.31	2.2
A. aerogenes	20	M	.62	5.2	12.5	.31	3.7	6.25	.31	2.4	1.25	.15	.9	25	.62	9.2
Klebsiellae	10	M	.62	2.7	12.5	.31	3.1	6.25	.31	2.3	1.25	.07	.6	25	.31	8.7
Protei	30	M	2.5	23.4	50	1.25	10.4	100	6.2	6.4	M	1.25	M	50	.62	5.2
Pseudomonades	20	M	12.5	M	M	1.25	71.4	M	12.5	67.2	5	.31	1.5	50	.62	15.5
Brucellae	12	M	2.5	20.5	12.5	.21	1.2	5	.15	1.0	5	.15	1.1	50	.62	4.0
Past. pestis	5	12.5	.62	2.1	6.25	.07	.4	5	.03	.3	2.5	.04	.2	1.25	.07	.6
V. cholerae	10	M	2	10.2	M	.07	5.1	M	.03	.23	M	.03	2.1	50	.07	1.9

Ma, Maximum; Mi, minimum; A, average.
M, More than 100 units or micrograms.
*End points expressed in micrograms per milliliter.
†Streptomycin-resistant strains.
‡Streptomycin-susceptible strains.

TABLE III. ACTION OF ANTIBIOTICS ON OTHER ORGANISMS IN VITRO*

ORGANISM	NUMBER OF STRAINS TESTED	PENICILLIN			STREPTOMYCIN			ENNIATIN			NEOMYCIN		
		MA	MI	A	MA	MI	A	MA	MI	A	MA	MI	A
Myco tuberculosis†	10	M	M	M	50	.01	4.9	20	5	10.9	2.5	.03	.5
Myco tuberculosis‡	7	M	M	M	M	M	M	15	10	20	1.25	.03	.25
Mycobacteria spp.	3	M	M	M	M	.01	7.6	50	5	35.2	6.25	.03	4.1
Bor. recurrentis	1		1.25			.2			M			.75	
Lept. icterohaemorrhagiae	1		.5			.4			M			.75	
E. histolytica	5										50	12.5	23.5
T. vaginalis	3										M	100	500
Sch. cruzi	1											1,000	
L. donovani	1											1,000	

Ma, Maximum; Mi, minimum; A, average.
M, More than 100 units or micrograms.
*Penicillin end points expressed in units per milliliter, all others in micrograms per milliliter.
†Streptomycin-susceptible strains.
‡Streptomycin-resistant strains.

TABLE IV. ACTION OF ANTIBIOTICS IN VIVO

ORGANISM	METHOD	STREPTOMYCIN	BACITRACIN	POLYMYXIN	ENNIATIN	NEOMYCIN
S. typhosa	5-day treatment	15 mg.	X	2.5 mg.	X	3 mg.
	1-day treatment	50 mg.	X	12 mg.	X	15 mg.
S. paratyphi B	5-day treatment	10 mg.	X	5 mg.	X	5 mg.
	1-day treatment	35 mg.	X	20 mg.	X	15 mg.
V. cholerae	5-day treatment	10.5 mg.	2,100 U.	6.1 mg.	X	7.5 mg.
Myco. tuberculosis	Mice, Youman's technique	L	L	L	4 mg.	3 mg.
Rickettsialpox	Guinea pigs	L	1,000 U.	1 mg.	X	2.5 mg.
Vaccinia	Rabbit's eye and mice	L	500 U.	L	L	>10 mg.
E. histolytica	Jones's technique	L	6,000 U.	X	X	32.5 mg.

L, LD_{50} of the antibiotic ineffective.
X, Not tested.

Corynebacterium diphtheriae (two fresh strains) was not inhibited by smaller than 100 microgram doses of any of the tested antibiotics. Neither were *Hemophilus influenzae* (one strain), *Neisseria meningitidis* (one strain), and *Neisseria gonorrhoeae* (one strain) influenced by neomycin.

Enterobacteriaceae reacted very favorably to neomycin. All streptomycin-resistant strains were checked by neomycin. This antibiotic has the advantage that both Protei and Pseudomonadaceae were inhibited by it. Brucellae and *Vibrio cholerae* strains, even those resistant to other antibiotics, were invariably checked by neomycin. Neomycin seems to have a broad range of activity in relatively small amounts against this group of organisms.

Both streptomycin-resistant and streptomycin-sensitive tubercle bacilli were tested. Neomycin proved to be strongly active against these bacteria, as well as against Mycobacteria isolated from so-called animal leprosy (Mycobacteria spp.). There was an inhibition of the growth of Treponemataceae and of *E. histolytica*. Two of the *Trichomonas vaginalis* strains also were checked by neomycin in large doses. Huge amounts of the antibiotic were necessary to inhibit *Schizotrypanum cruzi* and *Leishmania donovani*.

In animal experiments, neomycin in amounts equal to only one-half to one-fifth of the therapeutic dose of streptomycin was required to prevent clinically manifest lesions with Salmonellae. Also, polymyxin D showed a strong action in murine salmonellosis.

Neomycin was very efficient in preventing the development of cholera in mice. Both neomycin and enniatin III were effective against a streptomycin-resistant strain of *Myco. tuberculosis*.

Rickettsialpox did not cause fever in guinea pigs treated with daily doses of 100 mg. neomycin, or 10 mg. polymyxin or 1,000 U. bacitracin per kilogram.

Polymyxin D, bacitracin, and neomycin were ineffective against the viruses of herpes simplex, influenza, eastern and western equine encephalitis, and lymphocytic choriomeningitis. On the rabbit's eye, 500 U. of bacitracin applied not later than six hours after infection and repeated the second and third days thereafter prevented the development of lesions. Five milligrams of neomycin

instilled onto the cornea had a feeble action. While mice were protected by 200 U. per kilogram of bacitracin against vaccinia, 10 mg. per kilogram neomycin showed a favorable action in only 50 per cent of the animals.

E. histolytica did not cause amebic lesions in the intestines of young animals when they received an adequate dose of neomycin or bacitracin. It is, however, difficult to decide how much of this action is due to the inhibitory effect of these antibiotics on other intestinal organisms and how much has to be ascribed to the direct influence of these drugs on the protozoa themselves.

Maximal doses of neomycin, polymyxin D, and bacitracin did not prevent or cure *Sch. cruzi* and *L. donovani* infections in guinea pigs or *Tr. brucei* infections in mice.

DISCUSSION

The action of neomycin was tested on 370 organisms and the results were compared with those achieved with other antibiotics.

Neomycin has a favorable action on gram-negative rods, tubercle bacilli, and some gram-positive organisms, as well as on *E. histolytica* and rickettsialpox. Neomycin is much more active than streptomycin and compares favorably with other antibiotics. While polymyxin shows a stronger action on certain bacteria, neomycin might be preferred because of its lower toxicity if therapy by injections has to be considered. Enniatin III is irritating and, while strongly active against Mycobacteria, cannot be used in its present form in parenteral treatment.

SUMMARY

Neomycin showed strong action in vitro and in vivo on gram-negative rods, tubercle bacilli, *Bacillus anthracis, Listeria monocytogenes,* and intestinal protozoa. It had a variable action on gram-positive cocci. It checked blood flagellates in high concentration in vitro. In vivo, experimental salmonellosis, cholera, tuberculosis, amebiasis, and rickettsialpox could be prevented by neomycin in adequate doses. The activity of neomycin was compared with that of penicillin, streptomycin, Chloromycetin, aureomycin, polymyxin D, bacitracin, and enniatin III.

The authors are much indebted to Dr. Paul B. Szanto, Senior Pathologist of Cook County Hospital, for the histologic examinations of the experimental animals, and to Dr. William F. Schroeder and Mr. Chester L. Byrd, Jr., for their able help.

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RECOVERY OF HERPES SIMPLEX VIRUS FROM THE BLOOD OF A PATIENT WITH HERPETIC RHINITIS

ISAAC RUCHMAN, PH.D., AND KATHARINE DODD, M.D.
CINCINNATI, OHIO

INFECTION with the virus of herpes simplex is generally benign. The occurrence of systemic reaction or of occasional severe manifestations such as meningitis,[1] encephalitis,[2] or dermatitis[3] has led to the belief that there must be a viremia at some time during the course of the primary infection. Sporadic attempts in this laboratory to isolate a virus from the blood stream were unsuccessful until recently when the virus was recovered from the blood stream of a patient with a primary herpetic infection in the nose.

CASE REPORT.—A. G., an 8-year-old white boy, developed headache and fever two days before coming to the Children's Hospital Clinic on April 6, 1949. The child did not appear ill on admission to the clinic although his temperature was 101.3° F. In the left nostril there were tiny confluent vesicles surrounded by a red areola. The anterior cervical lymph nodes were enlarged. The tip of the spleen was palpable. There had been no previous history of fever blisters and no history of exposure to herpes. A swab of the nasal lesions transferred to the scarified corneas of rabbits induced keratoconjunctivitis. Blood drawn under aseptic conditions from the antecubital vein produced encephalitis in mice which were inoculated intracerebrally. The boy continued to have fever for eight days but at no time did he feel ill. There was no evidence of infection in the mouth or nose when he returned to the clinic on May 19, 1949. All animals inoculated at this time with swabbings from the boy's nasal mucosa and with his blood remained well.

RESULTS

Isolation of Virus From the Blood.—On the first clinic visit blood was obtained without anticoagulant and brought to the laboratory. Three hundredths milliliter of the blood-tinged serum was injected intracerebrally into each of five 18-day-old mice. When encephalitis developed in one animal on the third day a suspension of its brain was injected intracerebrally into five other mice. All five mice developed encephalitis and died four to fourteen days after inoculation. The strain was maintained for several passages. Of the remaining four mice inoculated with the patient's blood, one developed encephalitis on the third day and died two days later; two showed transient signs of encephalitis and were subsequently found to be immune to herpes. The fourth mouse remained well and was later not immune to herpes. Thus of five mice injected, two developed fatal infection and two were inapparently affected. Bacterial cultures were routinely performed throughout the investigation and were negative.

Isolation of Virus From the Nose.—A cotton swab moistened in saline solution was rubbed over the nasal lesions and applied to the scarified corneas of a rabbit. Keratoconjunctivitis developed in both eyes and a strain of virus was

From the Children's Hospital Research Foundation and the Departments of Bacteriology and Pediatrics of the University of Cincinnati College of Medicine.
Received for publication, Dec. 2, 1949.

recovered in mice by intracerebral subinoculation of a piece of the cornea. The strain of virus was maintained for several passages by mouse brain inoculation. The original cotton swab which had been kept in a refrigerator was examined at intervals for the presence of virus by corneal inoculation of rabbits. The rabbit eye test was positive successively at four, nine, thirteen, and twenty-one days and was negative thereafter. All animals that had developed keratoconjunctivitis were subsequently immune to intracerebral inoculation with the H. F. strain of herpes. The cornea of the rabbit tested at nine days showed the presence of herpetic inclusions.

Titration of A. G. Strains of Virus in Mice.—Intracerebral titrations were performed in young and old mice. The objective was threefold: (1) to obtain the titer of the freshly isolated strains, (2) to see if there was any difference in susceptibility between young and old mice, and (3) to obtain survivors for subsequent challenge with the homologous strain of virus. There were no marked differences in susceptibility between the young and old mice (Table I), although, contrary to expectation, the old mice which were inoculated with the strain from the blood appeared to be somewhat more susceptible than young mice. The titers were low, as was to be expected during early passages. After a few additional passages the titer was found to be much higher (see Table III).

TABLE I. TITRATION OF A.G. STRAINS OF VIRUS IN MICE INTRACEREBRALLY

STRAIN	PASSAGE	AGE OF MICE	0.03 ML. INTRACEREBRALLY				LD_{50} TITER*
			10^{-2}	10^{-3}	10^{-4}	10^{-5}	
A.G. (Nose)	3	20 days	–	2/4†	2/4	0/4	3.5
		3 mo.	3/5	3/5	3/5	–	3.4
A.G. (Blood)	4	17 days	–	2/4	1/4	0/4	3.2
		2 mo.	6/6	4/6	4/6	1/6	4.1

*Determined according to the method of Reed and Muench.[4]

†The numerator shows the number of mice that died; the denominator shows the number of mice inoculated.

IDENTIFICATION OF THE STRAINS OF VIRUS

Cross-Immunity Tests in Mice.—Proof of the identity of the infectious agents was established by performing series of resistance tests. Mice were first tested for cerebral resistance to the homologous strain of virus. The survivors were tested for immunity against heterologous strains a month later. The results are shown in Table II. Mice immunized with the H. F. and R. E.[3] strains of herpes virus were immune to the strains of virus recovered from the patient's blood and nose. Conversely, mice which had recovered from infection with the A. G. strains were solidly immune to challenge with the H. F. strain. Ten per cent suspensions of infected mouse brain were employed and tests were performed intracerebrally. All control mice succumbed.

Immunity Tests in Rabbits.—Rabbits that had recovered from ocular infection with the A. G. strains of virus were tested for immunity against the H. F. strain. Five rabbits that had been inoculated with the original swab from the patient's nose were immune to the H. F. strain injected intracerebrally. Similarly, a rabbit that had recovered from ocular infection with the strain from the blood was immune to cerebral infection with the H. F. strain.

TABLE II. CROSS-IMMUNITY TESTS IN MICE: STRAINS OF VIRUS ISOLATED FROM A.G. AND KNOWN STRAINS OF HERPES SIMPLEX VIRUS (H.F. AND R.E.)

EXPERIMENT	TESTED WITH VIRUS*	PASSAGE NUMBER	IMMUNIZED WITH†	RESULTS IN TESTED MICE‡
1	A.G. (nose)	5	H.F. herpes	0 0 0 0 0 0 0
			R.E. herpes	0 0 0 0
			Controls	3 3 3 3 3 3 3 4 4 5
2	A.G. (blood)	5	H.F. herpes	0 0 0 0 0 0 0 0 0 0
			R.E. herpes	0 0 0 0 0 0
			Controls	3 4 4 4 4 4 5 5 5 5 5
3	H.F. herpes	–	A.G. (nose)	0 0 0 0 0 0
			A.G. (blood)	0 0 0 0 0 0 0
			Controls	2 2 2 3 3 3

*Test dose consisted of 10 per cent suspensions of infected mouse brain either freshly prepared or frozen in solid carbon dioxide.

†Immunized mice had survived a previous intracerebral challenge with the homologous strain of virus.

‡Figure indicates day of death.

PRIMARY NATURE OF THE INFECTION DETERMINED BY NEUTRALIZATION TESTS

Portions of the acute and convalescent phase sera from Patient A. G. were heated at 56° C. for one half hour to destroy any virus that might be present and neutralization tests were performed with both the homologous strain of virus and the heterologous strains. The serum of a rabbit immunized against the H. F. strain of herpes was included.

The neutralization test was performed according to standard methods. Twenty per cent suspensions of infected mouse brain were prepared in skimmed milk and used the same day or stored in solid carbon dioxide until needed. Decimal dilutions of the virus were made in 20 per cent milk in saline and added to equal amounts (0.15 ml.) of undiluted test sera. The mixtures of virus dilution and serum were incubated for two hours in a water bath at 37° C. and then injected intracerebrally into groups of four mice each. Titers were computed according to the procedure of Reed and Muench[4] and neutralization indexes were determined by the method described in *Diagnostic Procedures for Virus and Rickettsial Diseases.*[5]

Table III shows the results. A definite rise in antibodies took place during convalescence against all three strains of herpes virus. The neutralization index against the homologous A. G. strain rose from 40 to 2,000 (fifty fold increase). The index increased from 2 to 320 (160 fold increase) against the H. F. strain and from 50 to 1,600 (thirty-two fold increase) against the R. E. strain. The immune rabbit serum prepared against the H. F. strain neutralized the A. G. strain with an index of 400. The index of this serum to the H. F. strain was 32.

SEARCH FOR VIRUS IN THE STORED BLOOD SERUM

The specimen of blood which had yielded the strain of virus was allowed to clot completely and after about two hours at room temperature the serum was drawn off and stored in solid carbon dioxide in a plastic tube. Seven weeks later the serum was thawed and inoculated intracerebrally into young dba and albino mice. All the mice remained well and at a later date were not immune to cerebral challenge with the homologous strain of herpes. The failure to recover

TABLE III. HOMOLOGOUS AND HETEROLOGOUS NEUTRALIZATION TESTS WITH ACUTE AND CONVALESCENT SERA OF PATIENT A. G.

VIRUS USED	SERUM IN TEST	DILUTIONS OF VIRUS						LD_{50} TITER*	NEUTRAL. INDEX†	INCREASE DURING CONVALESCENCE (FOLD)
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}			
A.G. (blood) 8th passage	Milk control	–	–	4/4‡	3/4	3/4	2/4	5.6	–	–
	H.F. imm. rabbit	4/4	4/4	2/4	0/4	–	–	3.0	400	–
	A.G. acute	–	4/4	4/4	2/4	0/4	–	4.0	40	–
	A.G. convalescent	3/4	3/4	1/4	0/4	–	–	2.3	2000	50
H.F. herpes	Milk control	–	–	3/4	1/4	0/4	–	3.5	–	–
	H.F. imm. rabbit	–	2/4	0/4	0/4	–	–	2.0	32	–
	A.G. acute	–	–	3/4	0/4	0/4	–	3.2	2	–
	A.G. convalescent	2/4	0/4	0/4	–	–	–	1.0	320	160
R.E. herpes	Milk control	–	–	–	4/4	4/4	2/4	6.0	–	–
	A.G. acute	–	–	3/4	2/4	2/4	0/4	4.3	50	–
	A.G. convalescent	4/4	3/4	2/4	0/4	–	–	2.8	1600	32

*Determined according to the method of Reed and Muench.[4]

†Calculated according to the method described in *Diagnostic Procedures for Virus and Rickettsial Diseases.*[5]

‡The numerator shows the number of mice that died; the denominator shows the number of mice inoculated.

the agent from the serum suggests (1) that after more prolonged contact with the serum the virus was inactivated or (2) that under conditions of storage the virus died off.

DISCUSSION

Attempts have been made in other laboratories to obtain a virus from the blood of patients with herpes. Negative results were reported by Lowenstein,[6] by Teissier and co-workers,[7] and by Schmidt.[8] Schmidt expressed the opinion that perhaps the virus may occasionally invade the blood stream. Bastai and Busacca[9] reported success in obtaining herpes virus from the blood stream of some patients with recurrent attacks of herpes, but their results are open to question because of the relative ease with which they were able to recover virus from their patients as well as from one of their controls. They also mentioned, without quoting the reference, the previous work of Veratti and Sala who were able to recover virus from the blood of four patients. Experimentally the virus may be found at times in the blood of inoculated rabbits.[10-12] It is found more regularly in the blood of infected mice.[13]

Recovery of the virus from the nose of the patient and the demonstration of a rise in antibodies during convalescence leaves no doubt that this was a primary infection with herpes. This case was unique in that the primary lesions were found in the nose. The finding of virus in the blood suggests several possibilities. (1) The virus might have been picked up by the needle from contaminated skin. This possibility was minimized by the absence of skin lesions, and by the fact that 70 per cent alcohol was used to disinfect the skin. In other patients where skin lesions were present on other parts of the body a contaminating virus was never picked up. (2) The virus might have been fortuitously present in the blood at the time. (3) The virus may be present early in the infection but may disappear rapidly from the blood stream. It has been observed that neutralizing antibodies against herpes reach their peak titer about a week

after initial symptoms appear (unpublished data). In the face of rapidly appearing antibodies the virus would probably soon disappear from the blood. Further investigation should furnish an answer to the problem.

Early attempts in this laboratory to find the virus in the blood of patients with primary herpetic infections were unfruitful. After the strain of virus was isolated from the blood of Patient A. G., stored sera from other patients with primary herpes were examined for the presence of virus. Inoculations into mice of acute phase sera which had been kept frozen in solid carbon dioxide yielded negative results. Freshly obtained samples of blood from individuals with proved primary herpetic infection were inoculated into mice without effect. Blind passages and tests for cerebral immunity failed to disclose the presence of virus in the original blood or serum inoculated. Some of these sera must have been obtained early in the disease since they were devoid of neutralizing antibodies.

SUMMARY

A strain of herpes simplex virus was recovered from the blood of a boy whose primary lesion was located in the nose. The patient developed neutralizing antibodies during convalescence against his own strain of virus as well as two known strains of herpes, thus indicating the primary nature of the infection. The strains of virus recovered were identified by the following methods. Mice immune to the patient's strains of virus were immune to known strains of herpes and, conversely, mice immune to known strains of herpes were immune to the strains isolated from the patient's blood and nose. Serum from a rabbit immune to herpes neutralized the virus recovered from the blood. Rabbits that had recovered from the infection with the human strains of virus were resistant to a known strain of herpes. Eosinophilic intranuclear inclusions of the herpetic type were demonstrated in the epithelium of the rabbits' cornea. Attempts to recover virus from fresh samples of blood and stored specimens of sera from other patients with primary herpetic infection yielded negative results.

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BRUCELLA STUDIES ON BANK BLOOD IN A GENERAL HOSPITAL

A. Agglutinins; B. Survival of Brucella

Wesley W. Spink, M.D., and Dorothy Anderson, B.S.
Minneapolis, Minn.

WHILE it would be highly desirable to prove the diagnosis of brucellosis culturally in every case, this is not possible in a large number of patients. From Aug. 1, 1937, to Aug. 1, 1949, several hundred suspected cases of brucellosis have been studied at the University of Minnesota and affiliated hospitals. During this time the diagnosis of active disease has been established in 158 cases. Brucella was isolated from the tissues or body fluids of 84, or 53 per cent, of the patients. The diagnosis of brucellosis in the patients with negative cultures was made on the basis of epidemiologic, clinical, and serologic evidence. In this clinic, considerable dependence has been placed upon the agglutination reaction for screening suspected cases of the disease and for establishing the diagnosis. In an epidemiologic study of brucellosis in Minnesota, it was shown in 267 culturally proved cases of brucellosis investigated consecutively from January, 1945, through June, 1948, that Brucella agglutinins were present at the time of isolation of the organisms in every patient except one.[1] In this one patient, agglutinins had been demonstrated in specimens of blood studied four and seven months previously. It was also observed that over 90 per cent of the patients had agglutinin titers of 1 to 320 or above. These epidemiologic studies further revealed that slightly over three-fourths of the patients were male.

In view of the significance attached to the agglutination reaction in the diagnosis of human brucellosis, it would be helpful to know the frequency with which agglutinins for Brucella occurred in an apparently healthy adult population drawn from the same endemic area of brucellosis. For comparative purposes, the same antigen and techniques employed in the diagnosis of brucellosis should be used in such a survey. An opportunity to carry out such a study arose at the University of Minnesota Hospitals in conjunction with the blood bank. Because this institution is a general hospital drawing patients from all sections of the state, donors of blood likewise come from all over the state, and mostly from rural areas. In addition, at least 80 per cent of the donors are adult males. Since there is evidence that Brucella in the blood of an asymptomatic donor can be transmitted to a susceptible individual through a blood transfusion, viability studies were made with the three species of Brucella and bank blood.[2]

METHODS OF STUDY

The present investigation was made in 1948 and involved a study of the blood of 1,627 apparently normal individuals. Blood was withdrawn from each donor and collected in commercially prepared containers* to which had been added an anticoagulant solution of

From the Division of Internal Medicine, University of Minnesota Hospitals and Medical School.

This investigation was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institute of Health, United States Public Health Service.

Received for publication, Dec. 8, 1949.

*Baxter Transfuso Vac, Baxter Laboratories, Inc., Morton Grove, Ill.

dextrose, sodium citrate, and citric acid. Each bottle or unit had 400 ml. of whole blood and 120 ml. of the anticoagulant. In collecting the blood from each donor, 10 ml. were placed in a small tube as a source of serum for performing the agglutination test.

The agglutination test was performed with an antigen prepared from *Brucella abortus*, strain 1193-3, and supplied by the Bureau of Animal Industry, United States Department of Agriculture, Beltsville, Md. Ten small tubes (12 by 100 mm.) were placed in a metal rack and to the first tube 0.9 ml. of physiologic saline solution and 0.1 ml. of serum were added and mixed. To each of the remaining nine tubes, 0.5 ml. of saline solution was added. Then 0.5 ml. of the diluted serum in the first tube was added to the second tube and mixed. From the second tube 0.5 ml. was removed and transferred to the third tube, and so on through each of the remaining tubes. Finally, 0.5 ml. of a saline suspension of the Brucella antigen was added to each of the tubes and thoroughly mixed with the diluted serum. A control tube contained 0.5 ml. of saline solution and 0.5 ml. of the antigen. The rack of tubes was placed in the water bath at 37° C. for twenty-four hours, and then each tube was examined for evidence of agglutination. The highest dilution of serum showing complete agglutination of the antigen was accepted as the titer of agglutinins for that serum. After this initial reading the rack was returned to the water bath and the tubes were examined after another period of twenty-four hours. Thus, the titer of agglutinins was determined after twenty-four and forty-eight hours of incubation.

To test the length of time during which Brucella might remain viable in citrated bank blood, collections of blood in the standard containers were made. Then 50 ml. of the whole blood were immediately transferred to each of six sterile bottles having a capacity of 100 milliliters. A representative strain of each of the species of Brucella was studied. These organisms were isolated from human sources and included *Br. abortus* Lynch No. 524, *Br. melitensis* No. 472, and *Br. suis* No. 374. The organisms were grown for twenty-four hours on slants of tryptose agar and then washed off and suspended in physiologic saline solution. The suspensions of Brucella were then seeded to the six bottles containing 50 ml. of blood. Two bottles were employed for each strain so that one contained approximately ten organisms per milliliter and the second about fifty per milliliter. The number of organisms was determined by making colony counts on inoculated tryptose agar plates. The mixtures of Brucella and citrated blood were stored in a refrigerator at 4° C. At appropriate intervals, 2 ml. of the blood were removed and placed in flasks of tryptose phosphate broth. These flasks were kept in an incubator at 37° C., and subcultures were made at the end of five, fifteen, and thirty days.

RESULTS

Agglutination Tests.—The results of the agglutination test for Brucella are presented in Table I. The values obtained demonstrate two significant facts.

TABLE I. BRUCELLA AGGLUTININ TITER IN 199 OF 1,627 BLOOD DONORS

DILUTION OF SERUM	POSITIVE TESTS AFTER INCUBATION FOR 24 HR.		POSITIVE TESTS AFTER INCUBATION FOR 48 HR.	
	NUMBER	PER CENT OF TOTAL	NUMBER	PER CENT OF TOTAL
1:20	104	6.4	126	7.74
1:40	57	3.5	107	6.57
1:80	21	1.3	42	2.58
1:160	9	0.55	14	0.86
1:320	5	0.31	8	0.49
1:640	2	0.12	3	0.18
1:1,280	1	0.06	1	0.06
1:2,560	0	0.00	1	0.06
Total number positive tests	199	12.24	302	18.54

First, agglutinins for Brucella were demonstrated in the blood of 199 donors, or 12.24 per cent, at the end of twenty-four hours of incubation of serum and antigen. With an additional twenty-four hours of incubation, agglutinins were present in the blood of 302, or 18.55 per cent. Since maximum values were obtained at the end of forty-eight hours of incubation, agglutination tests in this clinic are determined at the termination of this period. Second, most of the agglutinins found were present only in low titer. At the end of either twenty-four or forty-eight hours of incubation, the vast majority of bloods with agglutinins contained a titer of 1 to 80 or less. If the maximum values of forty-eight hours of incubation are selected, only twenty-seven individuals or 1.66 per cent had Brucella agglutinins in a dilution of 1 to 160 or higher. It has already been pointed out that in culturally proved cases of burcellosis over 90 per cent of the patients had simultaneous agglutination titers of 1 to 320 or more. In the present study of presumably normal individuals, less than 1 per cent had a comparable titer.

The next inquiry was directed to the significance of the presence of Brucella agglutinins in this normal group. The most obvious explanation was that since the individuals lived in an endemic area of brucellosis, the agglutinins reflected asymptomatic invasion of the tissues by Brucella, or the individuals had had attacks of brucellosis that were either disregarded or misdiagnosed. Another possibility was that Brucella antibodies might have been stimulated following the ingestion of killed Brucella present in either pasteurized or raw milk.[3] It is well established that Brucella agglutinins may appear following stimulation of the tissues by antigenically related microorganisms such as *Pasteurella tularensis*. This phenomenon also accounts for the appearance of Brucella agglutinins following vaccination with cholera vaccine.[4] However, there is little evidence in this normal group that *Past. tularensis* or *Vibrio comma* played a significant role in the causation of Brucella agglutinins. In order to obtain more precise information about the possible exposure of the donors to brucellosis, a letter was sent to each of the twenty-seven individuals whose serum revealed a maximum titer of agglutinins of 1 to 160 or above. Information was requested concerning their contact with cattle and hogs; whether they had had contact with cattle having Bang's disease; if they had ingested raw milk; whether they had been ill at all during the past two years; and if they had been in military service, whether they had been immunized against cholera. Replies were received from ten of the twenty-seven donors, all of whom were men. This information is summarized in Table II. It is significant that two of the three individuals with titers of 1 to 320 and the two with values of 1 to 640 had good histories of contact with potential sources of the disease. Donor 2 may actually have had active disease at the time his blood was drawn. It is possible that immunization for cholera may have induced the titer of 1 to 160 in Donor 4.

Survival of Viable Brucella in Bank Blood.—Four experiments were carried out to determine the length of time Brucella survived in refrigerated citrated blood. In the first experiment, organisms from each of the three species were successfully subcultured every day for the first week. This applied to an inoculum of both ten organisms and fifty organisms per milliliter. The in-

TABLE II. DATA ON "NORMAL" BLOOD DONORS WITH ELEVATED TITER OF BRUCELLA AGGLUTININS

DONOR	TITER OF AGGLUTININS	OCCUPATION	CONTACT WITH ANIMALS	PROBABLE SOURCE OF INFECTION	HISTORY OF ILLNESS
1	1:160	Not known	No	Not known	Yes. Fever 2 yr. before, undiagnosed
2	1:160	Machinist	No	Not known	Yes. Fever, chills; sweats at present
3	1:160	Not known	No	Visit to farm where Bang's disease existed	No illness
4	1:160	Farmer	Yes	Not known	Immunized against cholera in Army
5	1:160	Farmer	No	Not known	No illness
6	1:320	Formerly farmer and packing plant employee	Yes	Not known	None, except drowsiness for 2 yr.
7	1:320	Not known	No	Drinks raw milk	None
8	1:320	Farmer	Yes	Bang's disease in cattle	No
9	1:640	Beef boner, packing plant	Yes	Not known	No
10	1:640	Farmer	Yes	Bang's disease in cattle	"Only bad colds"

oculated blood contained no agglutinins for Brucella. In the second experiment, also performed with blood having no Brucella agglutinins and with the same number of organisms, Brucella of the three species were isolated daily for the first week and then at the termination of fourteen and fifty-eight days. There was insufficient blood available to determine if *Br. abortus* survived longer than the fifty-eight days, but *Br. melitensis* and *Br. suis* were cultured from the blood after remaining in the refrigerator for 133 days. In a third experiment, ten and fifty colonies of each of the three species were seeded to citrated blood having no Brucella agglutinins and then placed in the refrigerator for three months. At the conclusion of this period, Brucella of the three species were cultured from all the inoculated flasks of blood. These experiments were carried out with blood obtained from normal individuals who had no evidence of brucellosis. It might be predicated on this basis that Brucella might survive in blood from a healthy or "nonimmune" individual but not in the blood of a patient who has the disease, or is recovering from brucellosis. Therefore, blood was collected from a young adult male subject who had had acute brucellosis for two weeks before he entered the hospital. During the first week of observation, *Br. abortus* was isolated from the patient's blood on four different days. During the fourth week of illness, treatment with aureomycin was started and continued for ten days. Coincident with this the patient improved considerably. Subsequent blood cultures remained sterile, and the patient left the hospital feeling well. One month after treatment with aureomycin, blood was drawn for viability studies. The blood was sterile, and Brucella agglutinins were present in a titer of 1 to 2,560. The results with the use of this blood are presented in Table III. It is to be noted that organisms in the larger inoculum survived for as long as six months. Even the smaller inoculum of all three species remained viable for at least two months.

TABLE III. LENGTH OF SURVIVAL OF EACH OF THREE SPECIES OF BRUCELLA IN WHOLE CITRATED BLOOD FROM A PATIENT CONVALESCING FROM ACUTE BRUCELLOSIS

STRAIN	INOCULUM	MONTHS OF REFRIGERATION AND RESULTS OF SUBCULTURES						
		1	2	3	4	5	6	7
Br. abortus Lynch No. 524	10 cols./ml.	+	+	0	0	0	0	0
	50 cols./ml.	+	+	+	+	+	+	0
Br. melitensis No. 472	10 cols./ml.	+	+	0	0	0	0	0
	50 cols./ml.	+	+	+	+	+	+	0
Br. suis No. 374	10 cols./ml.	+	+	+	+	0	0	0
	50 cols./ml.	+	+	+	+	+	0	0

+, Growth.
0, No growth.

DISCUSSION

Since the agglutination test is used so widely for the diagnosis of brucellosis, it is essential that any given laboratory should employ a reliable and sensitive antigen with a standardized technique. Brucellosis exists for the most part as a sporadic disease. In the interpretation of a positive agglutination reaction, the titer of agglutinins has considerable clinical significance. This is borne out by the experience at the University Hospitals. To recapitulate, no patient with culturally proved acute or chronic brucellosis has been encountered with absent agglutinins, and, with one exception, the titer of agglutinins in the proved cases has been 1 to 160 or higher. On the other hand, the diagnosis of active disease has been made on many occasions in patients with epidemiologic and clinical evidence of brucellosis, where the titer of agglutinins was 1 to 160 or greater. The present study on the agglutination reaction in presumably healthy blood donors living in an endemic area of brucellosis indicates quite clearly that a relatively large number of individuals possess a low titer of agglutinins. Referring to Table I, it is seen that on the basis of an agglutination test with twenty-four hours of incubation, 9.9 per cent have agglutinins in titers up to 1 to 40. After forty-eight hours of incubation of serum and antigen, 14.31 per cent have similar titers. With higher titers, the number of positive reactions diminishes considerably, although almost 3 per cent have a titer of 1 to 80. When titers of 1 to 160 or higher are reached, only 1 to 2 per cent have agglutinins in this range. While many individuals are of the opinion that cases of chronic brucellosis occur commonly in which agglutinins are absent, such statements are rarely accompanied by bacteriologic proof of active disease. Until such evidence is forthcoming, caution should be exercised in establishing the diagnosis when repeated observations show an absence of agglutinins. This skepticism should be further extended to patients having a titer of Brucella agglutinins below 1 to 100.

The widespread use of citrated whole blood for human transfusions poses the problem of the possible transmission of Brucella through the blood of an asymptomatic but bacteremic individual. The present study has shown that an inoculum of relatively few Brucella of all three species will remain viable in refrigerated bank blood for several months. This applies not only to the blood

of healthy individuals without evidence of brucellosis, but also to the blood of a patient who had apparently recovered from an acute attack. In immunologic studies carried out by Hall,[5] organisms remained viable in citrated blood from chronically ill patients. While organisms may remain viable for several months in citrated blood kept at 4° C., Hall[5] also has shown that Brucella do not survive for longer than four hours when the blood is kept in an incubator at 37° C. From a practical point of view the possibility is remote of transmitting brucellosis from human to human by means of blood transfusions. If only healthy donors of blood are selected, there would be remarkably few individuals having a bacteremia without symptoms of the disease. Of considerable importance is the fact that patients having brucellosis with positive blood cultures usually have relatively few organisms in their blood as determined by colony counts. This feature was pointed out by members of the Mediterranean Fever Commission, in their studies on the epidemiology of brucellosis.[6] Even if blood from the rare donor with a bacteremia were to be introduced into a patient, it is not unlikely that the Brucella would be destroyed by the blood or tissues of the recipient. Furthermore, most patients with fever of unknown etiology in a hospital, ill enough to receive transfusions, usually receive one or more of the many available antibacterial agents, which would lead to further destruction of the organisms.

SUMMARY

1. Brucella agglutinins were demonstrated in the blood of 302 (18.55 per cent) of 1,627 apparently healthy donors of blood to a blood bank in a general hospital. Only 27 or 1.66 per cent had agglutinin titers of 1 to 160 or higher. There was some evidence that the donors with the high titers had had contact with the disease in the past.

2. Since even in an endemic area, only 1 or 2 per cent of a normal population has Brucella agglutinins in a significant titer, the agglutinin reaction is a valuable procedure in the detection of active disease.

3. When small numbers of viable Brucella of the three species are seeded to citrated whole blood, organisms may be cultured as long as six months after, if the blood is refrigerated at 4° C.

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LABORATORY METHODS

AN INDIRECT, QUANTITATIVE METHOD FOR THE ESTIMATION OF HEPARIN ACTIVITY IN VITRO: THE HEPARIN-PROTAMINE TITRATION TEST

GEORGE V. LEROY, M.D., BERNARD HALPERN, M.S., AND RALPH E. DOLKART, M.D.
CHICAGO, ILL.

INTRODUCTION

THE existence of an anticoagulant substance in normal mammalian blood was first demonstrated by Howell[1] in 1923. He identified this material as "heparin" and isolated it from various organs, in particular the liver. On the basis of his studies he concluded that heparin functioned as an antiprothrombin, and to a lesser extent as an antithrombin. The chemical nature of heparin has been studied extensively by Jorpes[2] who has shown that it is a mucoitin sulfuric acid ester, that it occurs in all mammalian tissues studied, and that it is a very stable and chemically active substance. The precise site of production of heparin has been studied by Holmgren and Wilander[3a] and others. It is now generally accepted that heparin is elaborated in the mast cells of Ehrlich. These cells are sometimes called "heparinocytes." The occurrence of these cells in granulation tissue, in close association with newly formed blood vessels, is of great interest. Jaques and Waters[3b] found that anaphylactic shock in dogs, which renders the blood incoagulable, is accompanied by an excess of heparin in the blood. Ehrich and co-workers[4] measured the local concentration of heparin produced by human mast cells in a patient with elephantiasis of the scrotum. Allen[5a] and his colleagues reported that heparin was found in dog's blood which had become incoagulable after a single exposure to 450 r of 250 kv. x-ray delivered to the whole body. These workers demonstrated the existence of heparin by the use of known antiheparin agents: toluidine blue and protamine sulfate. They were able[5b] to extract heparin as the amorphous Ba salt from the blood of irradiated dogs. Cronkite[6] studied the blood of swine exposed to lethal doses of gamma radiation from the atomic bomb at Bikini. He was able to reduce the prolonged clotting time in seven of ten animals by the use of toluidine blue. Holden and associates[7] were not able to detect heparin in the blood of dogs exposed to total body roentgen irradiation. Jacobson and co-workers[8] demonstrated heparinemia in rabbit's blood after the administration of repeated doses of the nitrogen mustard, methyl-*bis* (β-chloroethyl) amine hydrochloride. A similar observation has been made in the case of patients under treatment with this material.[8]

The specificity of the reaction of toluidine blue or protamine sulfate with heparin is well known, and there is no convincing evidence that either of these agents can shorten the clotting time of whole blood by any other means than

From the III Medical Service Laboratory, St. Luke's Hospital.

These studies were aided by a grant from the Commercial Solvents Corporation, New York, N. Y., and the W. D. Monroe Fund, Chicago, Ill.
Received for publication, Oct. 26, 1949.

the neutralization of excessive amounts of heparin which may be present. Allen and associates[5c, d] have reported the use of toluidine blue and protamine sulfate in a variety of hemorrhagic states where laboratory studies suggest the existence of heparinemia. Holonbek, Hendrick, and Hollis[10] have described the successful use of toluidine blue in the management of similar patients. LeRoy, Halpern, Stone, and Dolkart[11] have used protamine sulfate to advantage in the treatment of a large group of patients with hemorrhagic states.

The empirical nature of most of these laboratory and clinical studies has been a source of concern to all who have studied this problem. Uncertainty as to the actual occurrence of heparin in human blood has been opposed by the indubitable finding that certain samples of hypocoagulable blood can be caused to clot normally or more quickly after the addition of either of the two well-known antiheparin agents, and by the clinical observation of the cessation of bleeding immediately after the administration of appropriate amounts of these drugs. Recently Jaques[12] has reported the recovery of the brucine salt of heparin from normal human blood in small amounts. Parkin and co-workers,[13] from the Mayo Clinic, were unable to confirm the finding of abnormal protamine titration tests in human beings with hypocoagulable blood. Best and Limarzi[14] have reported variable results using the whole blood technique described by Allen. Carr and Fowler[15] have reported that the blood of patients with thrombocytopenic purpura is abnormally sensitive to the anticoagulant action of heparin. Conley and associates[16] have found that removal of platelets from plasma renders it more sensitive to the effect of heparin. The technical difficulties involved in the isolation of heparin from blood are too great to permit the use of this method in clinical and routine laboratory practice. Indirect methods are described which will allow one to estimate (if possible) the extent of the effect of endogenous heparin on the clotting mechanism. A test which will permit one to conclude that blood, or other material, contains an anticoagulant effectively equivalent to "x gamma of heparin per milliliter of plasma" is described in this report.

The action of heparin on blood clotting is difficult to evaluate because of the many factors that influence the rate of clot formation. Human whole blood clotting time may vary from a few minutes to many hours depending on the technique used in handling the blood. When blood is handled in such a way that the extraneous factors are relatively constant, the effect of the addition of heparin is characteristic. Increasing concentrations of heparin cause a prolongation of the clotting time which is exponential over a range of concentrations of approximately 10 gamma per milliliter to 60 gamma per milliliter.* This reaction of the whole blood clotting time to the addition of heparin has been exploited clinically in the so-called heparin tolerance tests. These tests have been planned to demonstrate the presence of clot-promoting factors in blood such as may occur in the course of thromboembolic diseases. De Takats[17] devised a heparin tolerance test in which heparin is given intravenously, and the clotting time of whole blood is estimated by the capillary tube method. It was postulated

*Gravimetric values for heparin refer to material with an activity of 110 units per milligram unless otherwise specified.

that the blood of patients with thromboembolic diseases would be affected to a lesser extent than normal, i.e., would tolerate heparin better. Waugh and Ruddick[18] applied the same principle to an in vitro test in which whole blood is added to varying concentrations of heparin and the clotting time determined. The results of the in vivo and the in vitro heparin tolerance tests are not entirely parallel, but the variations are probably due to extraneous factors resulting from the handling of the blood. Following the same general line of reasoning, Allen and associates[5c] developed a protamine titration test in which whole blood is rendered incoagulable by the addition of a fixed amount of heparin per milliliter. Varying amounts of protamine sulfate (or toluidine blue) are then added to *titrate* the total (i.e., the endogenous and the added) heparin content of the blood. It was their observation that more protamine sulfate (or toluidine blue) is required to cause rapid (normal) clotting in certain hemorrhagic states than in the controls. This increased coagulation-resisting activity can be attributed to the presence in such whole blood of an amount of endogenous heparin sufficient to contribute to the production of the hemorrhagic state, but insufficient to be detected by any other known method.

These workers[5f] performed control observations which satisfied them that the phenomenon observed was not due to excessive heparin cofactor or to alterations of the Ac-globulin content of the blood concerned. The test to be described in this paper is based on the foregoing considerations, but uses oxalated plasma which we believe is more stable and less affected by handling than is whole blood. There are three major objections to these whole blood techniques:

(1) The character of the glassware and the technique of handling the blood introduce a series of variables which we could neither predict nor control.

(2) Time and temperature become important factors with whole blood, since it is desirable to perform the test as soon after venipuncture as possible. Where blood must be carried from the hospital to the laboratory, additional variables are introduced.

(3) Allen's first technique[5d] required the use of 250 gamma heparin per milliliter of blood, and his second technique[5e] used 90 gamma heparin per milliliter of blood. Assuming that Jaques's[12] value of 1.0 gamma per milliliter represents approximately the normal state, the ratios of added to endogenous heparin seemed undesirably high. For these reasons we decided to work with oxalated plasma and to determine the clotting time of this material in a manner similar to that known as the one-stage prothrombin time test. In this way it became possible to control most of the variables, and to work with amounts of added heparin which were not many times greater than the normal concentrations reported by Jaques.

A variety of methods have been devised for the assay of pharmaceutical preparations of heparin. The ones currently in use are based on a Provisional International Standard of heparin, the Na salt of which has an activity of about 130 units per milligram. In the United States most commercial heparin preparations have an activity of 110 units per milligram. When the Provisional

International Standard or the Connaught Laboratories' heparin[16] is used for a reference material, the test to be described can be used for quick and accurate routine assay of materials containing heparin.

METHOD

The procedure to be described is a modification of the one-stage prothrombin time test. It is important in tests of this type to introduce as few variables as possible. The total volume of the test mixture, the concentration of thromboplastin, and the concentration of $CaCl_2$ solution are kept constant. The final dilution of oxalated plasma in the test mixture is 20 per cent (1:4). It is important to use oxalated plasma which has not been removed from the tube in which the oxalated blood was centrifuged. When separated plasma is used in clotting studies, a significant variation in results will be noted over a period of four to twenty-four hours. When oxalated plasma is allowed to remain in contact with the cells, the clotting time varies less than 5 sec. (20 per cent) over a period of several days, even if the blood is stored in a refrigerator at 0 to 5° C. In the discussion which follows, the time in which coagulation occurs after the addition of thromboplastin-$CaCl_2$ is referred to as the *clotting time*. The term *prothrombin time* is avoided since variations in *time* result from the conditions of the test. With our method, the usual values for clotting time are 18 to 22 seconds. Repeated determinations using the same sample of oxalated plasma should agree within 0.5 second. When the test is performed properly the addition of heparin should affect the clotting time of oxalated plasma in an exponential manner. (See Fig. 2.)

Technique of One-Stage Clotting Time Test.—The glassware should be Pyrex, throughly cleaned with acid-dichromate, rinsed repeatedly with distilled water, and dried in an oven. Four and five tenths milliliters of blood are added to 0.5 ml. of 0.1M sodium oxalate solution. The blood is centrifuged at 2,000 r.p.m. for five minutes. The oxalated plasma is not separated. One-tenth milliliter of oxalated human plasma is placed in a 12 by 75 mm. test tube in a water bath kept at 36.5 ± 0.5° C.; 0.1 ml. of 0.85 per cent solution of sodium chloride (salt solution) is added, and the tube agitated for mixing. A second portion of 0.1 ml. of salt solution is added, agitated, and immediately 0.2 ml. of thromboplastin-$CaCl_2$ solution is blown from a pipette into the tube. At the same time, the electric timer is started. The tube is swirled gently in the water bath for 9.0 seconds. It is then held over a well-lighted concave mirror and agitated gently each second. At the first appearance of fibrin threads or gel-formation the timer is stopped and the time recorded. When solutions of heparin and/or protamine sulfate are employed in the test, they replace one or both of the 0.1 ml. portions of salt solution. When both heparin and protamine sulfate are used, the heparin solution is added first. In this way the volume of the test mixture is kept at 0.5 milliliter.

Titration of Thromboplastin.—Preliminary studies showed that when Maltine thromboplastin solution was diluted, there was little influence on the clotting time until concentrations of 20 to 10 mg. per milliliter were reached (2 per cent to 1 per cent). When the concentration of Maltine thromboplastin is plotted against the clotting time, the curve usually shows an upward turn at this point. It is desirable to use the concentration of thromboplastin just before the break in the curve. A series of dilutions of Maltine thromboplastin are prepared. Each sample is stirred constantly in a water bath at 56° C. for ten minutes. It is then cooled to room temperature, and an equal volume of 0.025M solution of $CaCl_2$ is added. This mixture is stirred for four minutes, and then centrifuged at 2,000 r.p.m. for four minutes. The supernatant is decanted for use in the test. The results of a typical titration curve for Maltine thromboplastin are shown in Fig. 1. The concentration which is most suitable is indicated by the arrow. Once a batch of thromboplastin has been titrated it is not necessary to repeat this operation. A sufficient quantity of Maltine thromboplastin of the same batch can usually be obtained to last a year. The thromboplastin-$CaCl_2$ mixture should be made up fresh each day.

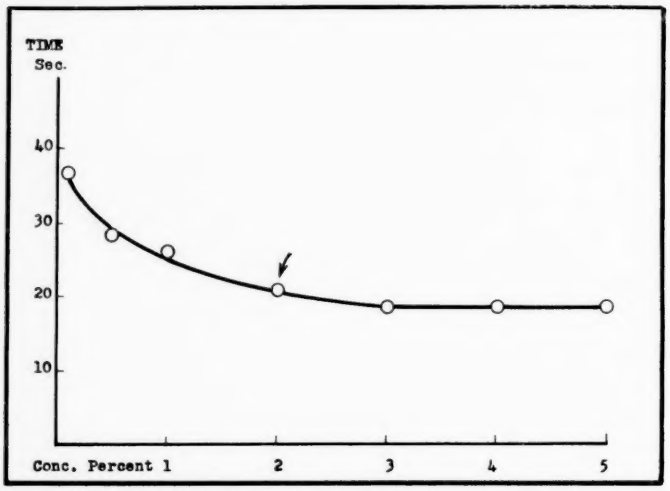

Fig. 1.—Effect of thromboplastin concentration on clotting time.

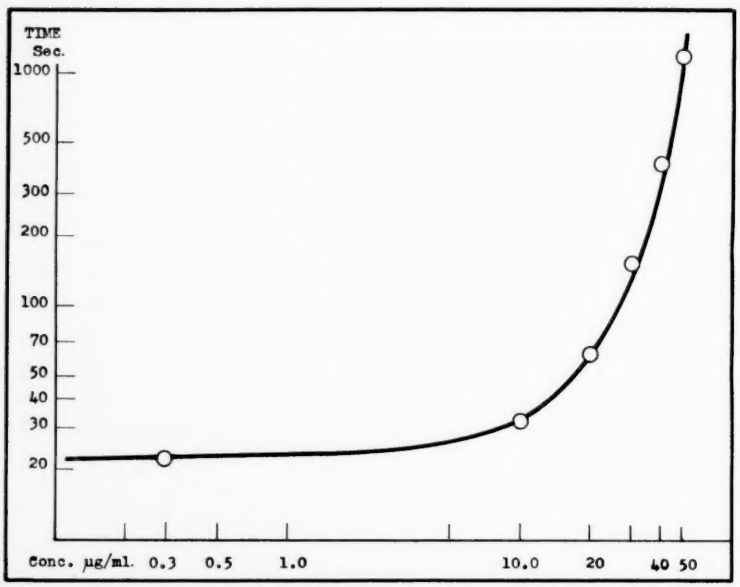

Fig. 2.—Effect of heparin on clotting time.

This curve was analyzed mathematically, and its formula was found to be: $y = 17 + 4.85\ (1.115)^{x}$, where y = clotting time in seconds; and x = concentration of added heparin in gamma per milliliter. The values used were as follows:

Added Heparin	C. T. (Sec.)
10	32
20	60
30	150
40	400
50	1100
0	22

According to the equation, the clotting time which should be found if no heparin were added is 21.85 seconds. The control clotting time for this plasma was 22 seconds. Applying the equation, it is found that the heparin concentration x for this value of y is about 0.3 to 0.6 gamma per milliliter. (We are indebted to Mr. Theodore Fields, Radioisotope Unit, Veterans Administration Hospital, Hines, Ill., for this analysis.)

Titration of Calcium Chloride Solution.—There is sufficient variation in the effect of $CaCl_2$ concentration on the clotting test to justify titrating this reagent for the particular batch of thromboplastin that is used. Using the optimum concentration for thromboplastin (as above), a titration is made of the effect of $CaCl_2$ solutions varying in strength from 0.1M to 0.01M. The optimum concentration of $CaCl_2$ is generally 0.02M or 0.025M.

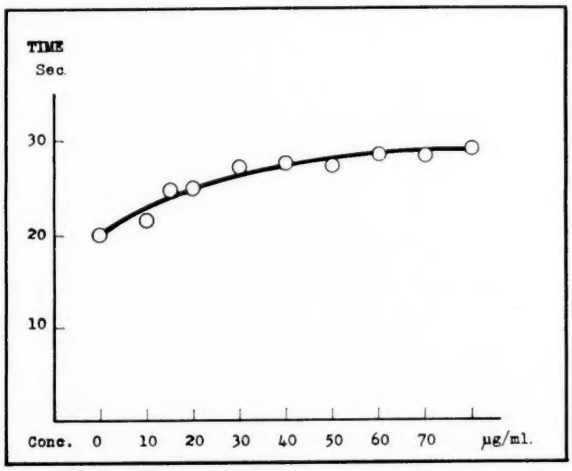

Fig. 3.—Effect of protamine sulfate on clotting time.

Effect of Heparin and Protamine Sulfate Added Independently to the One-Stage Clotting Time System.—When increasing concentrations of heparin or protamine sulfate are added to the clotting time system, a typical prolongation of clotting time occurs. The effect of Connaught Laboratories' heparin is shown in Fig. 2. The effect of protamine sulfate solution is shown in Fig. 3. It is not necessary to perform these tests as a preliminary to the heparin-protamine titration to be described.

The Heparin-Protamine Titration Test.—A working standard of Connaught Laboratories' heparin (95 units per milligram) is prepared containing 1.15 mg. per milliliter of salt solution. Since most commercial heparins have an activity of 110 units per milligram, this solution is equivalent to 1,000 gamma "active heparin" per milliliter. Dilute solutions containing 10, 20, and 30 gamma per milliliter respectively are prepared from the standard. These solutions may be stored at –5° C. to –15° C., and should be renewed every two to three weeks.

A working standard of protamine sulfate (Lilly) containing 1,000 gamma per milliliter of salt solution is prepared, and dilute solutions are made up containing 10, 15, 20, 30, 40, 50, 60, 70, and 80 gamma per milliliter respectively. These, too, may be stored at –5° C. to –15° C. for not longer than three weeks. It is our practice to use protamine sulfate powder, and not the 1 per cent solution of protamine sulfate (Lilly). If the latter is used it is necessary to assay each batch, using a standard heparin solution, and adjust the volumes accordingly.

Eight or ten test tubes containing 0.1 ml. of oxalated plasma are placed in the water bath. The first tube is the control and 0.2 ml. of salt solutions is added to it. One-tenth milliliter of one of the dilute heparin solutions is added to each of the remaining tubes. Then 0.1 ml. portions of each of the concentrations of dilute protamine sulfate solution are added to all but one of these tubes. After blowing in thromboplastin $CaCl_2$ solution, the clotting time is determined on each mixture in turn. A typical experimental setup is shown in Table I.

TABLE I. TYPICAL EXPERIMENTAL SETUP FOR HEPARIN-PROTAMINE TITRATION TEST

TUBE	CONCENTRATION IN GAMMA/ML.	
	HEPARIN SOLUTION	PROTAMINE SOLUTION
1	0	0
2	10	0
3	10	10
4	10	15
5	10	20
6	10	30
7	10	40
8	10	50, etc.

Tube 1 gives the normal control clotting time of the oxalated plasma sample. Tube 2 demonstrates the magnitude of the anticoagulant effect of heparin on the sample. This result should be compared with the curve in Fig. 2. In the remaining tubes, the most rapid clotting time will occur in the one in which the heparin is just completely neutralized by the protamine sulfate. This time is not necessarily identical with the control clotting time (Tube 1), but it is always close to it.

The ratio of protamine sulfate to heparin necessary for this neutralization has been consistently 1.5:1 in our laboratory. This ratio appears to be a true biologic constant when this one-stage clotting time test of oxalated plasma is used. It is our practice to perform the test two times on each sample of oxalated plasma, using either 10 and 20 or 20 and 30 gamma per milliliter heparin solutions. These two tests provide a good internal check. Calculations of the amount of excess heparin in each test should yield similar values.

Typical results obtained with oxalated plasma from fifteen normal human subjects are shown in Tables II, III, and IV. The clotting times for the tubes in which the characteristic neutralizing ratio of 1.5 parts of protamine sulfate to 1 part of heparin occurred are shown in boldface type.

TABLE II. HEPARIN-PROTAMINE TITRATION TESTS IN NORMAL SUBJECTS

THE CONCENTRATION OF THE HEPARIN SOLUTION USED WAS 10 GAMMA/ML. NOTE THAT THE PROTAMINE:HEPARIN RATIO IS 1.5:1 IN EVERY CASE (BOLDFACE NUMBERS)

TUBE*	CONCENTRATION PROTAMINE (GAMMA/ML.)	CLOTTING TIME IN SECONDS FOR SUBJECT					
		1	2	3	4	5	6
1	0	21.7	22.0	22.0	20.0	20.7	21.5
2	0	33.6	40.1	36.4	31.0	30.2	32.3
3	10	25.2	26.9	26.9	23.0	24.0	24.2
4	15	**24.0**	**24.2**	**24.8**	**21.6**	**22.5**	**23.0**
5	20	25.3	26.6	28.7	23.2	25.7	24.3
6	30	28.5	32.8	33.0	25.4	29.3	26.7
7	40	32.8	36.3	41.3	28.4	33.1	29.5
8	50	38.1	38.6	39.6	31.7	33.6	32.3

*Tubes set up as in Table I.

TABLE III. HEPARIN-PROTAMINE TITRATION TEST IN NORMAL SUBJECTS

THE CONCENTRATION OF THE HEPARIN SOLUTION USED WAS 20 GAMMA/ML. NOTE THAT THE PROTAMINE:HEPARIN RATIO IS 1.5:1 IN EVERY CASE (BOLDFACE NUMBERS)

TUBE	CONCENTRATION PROTAMINE (GAMMA/ML.)	CLOTTING TIME IN SECONDS FOR SUBJECT					
		7	8	9	10	11	12
1	0	19.9	20.8	21.0	22.0	21.0	19.3
2	0	42.5	28.9	68.8	70.0	95.0	63.0
3	10	32.3	27.0	39.5	59.0	31.5	55.0
4	20	27.7	23.7	24.1	35.9	26.1	30.6
5	30	**24.6**	**21.8**	**22.9**	**24.0**	**22.9**	**21.0**
6	40	27.7	23.7	28.9	30.0	28.3	31.1
7	50	32.9	27.2	32.0	31.5	30.4	34.4
8	60	36.9	28.2	31.0	32.4	30.0	36.8

TABLE IV. HEPARIN-PROTAMINE TITRATION TEST IN NORMAL SUBJECTS
THE CONCENTRATION OF THE HEPARIN SOLUTION USED WAS 30 GAMMA/ML. NOTE THAT THE PROTAMINE:HEPARIN RATIO IS 1.5:1 IN EVERY CASE (BOLDFACE NUMBERS)

TUBE	CONCENTRATION PROTAMINE (GAMMA/ML.)	CLOTTING TIME IN SECONDS FOR SUBJECT		
		13	14	15
1	0	19.6	22.0	20.8
2	0	113.6	88.0	200.0
3	10	115.0	---	---
4	20	99.1	50.0	55.8
5	30	34.3	38.1	34.7
6	40	24.7	29.4	26.1
7	45	**21.4**	**26.1**	**24.4**
8	50	23.2	27.3	25.9
9	60	26.6	30.7	26.2

It is possible to detect the effect of the addition of as little heparin as 3.3 gamma per milliliter of oxalated plasma by this method. This amount of heparin is approximately equivalent to 1.7 gamma per milliliter of whole blood. In ordinary clinical practice when specimens of blood are tested which are known or suspected to contain excessive heparin, the results of the heparin-protamine titration are abnormal. It is characteristic of these bloods that when heparin is added to oxalated plasma in x amounts, more than 1.5 x protamine sulfate is required to cause the most rapid clotting. Likewise, the effect on the clotting time of the addition of heparin in concentrations of x gamma per milliliter is greater than with normal specimens. It is our basic assumption that excess protamine sulfate required to cause the most rapid clotting is consumed in neutralizing an excess of heparin. The ratio of excess protamine sulfate to excess heparin to cause complete neutralization is 1.5:1, the characteristic ratio. Thus, if it is found that a sample of oxalated plasma to which heparin has been added in amounts of 10 gamma per milliliter requires 20 gamma per milliliter of protamine sulfate, it is concluded that this plasma originally contained an additional 3.3 gamma of heparin per milliliter: 5 gamma excess protamine $\div$ 1.5 = 3.3 gamma of heparin.

When heparin-protamine titration tests are performed on normal subjects shortly after the administration of 50 mg. of heparin intravenously, abnormal ratios can be demonstrated, and the excess heparin in the blood can be estimated. The results of one such experiment are shown in Table V. Subject A. B. weighed 70 kilograms; assuming a normal value for plasma volume, the heparin content of the plasma should be 15 gamma per milliliter. In Table V, B, it is seen that the amount of heparin found was 10 gamma per milliliter, that is, an excess of 15 gamma per milliliter protamine sulfate was required. It should be noted that this value was found when the oxalated plasma was titrated with protamine sulfate alone, as well as when the heparin-protamine titration was performed. In some instances, simple titration with protamine sulfate solution will demonstrate the existence of heparinemia (see Table V, B). This is particularly true when one is dealing with blood obtained from a patient who has received heparin parenterally. In the case of patients with heparinemia of endogenous origin, it is generally necessary to perform the complete heparin-protamine titration test as described. At the present time it is a matter for speculation why it is necessary to add heparin to oxalated plasma apparently containing an excess of endogenous heparin in order to demonstrate the presence of the latter.

In practice it is rarely possible in the test to use concentrations of added heparin as great as 30 gamma per milliliter when one is dealing with oxalated plasma containing excess "endogenous" or therapeutically administered heparin in concentrations greater than 6.6 gamma per milliliter. Under these circumstances the prolongation of the clotting time exceeds 100 to 150 seconds. We proceed now to clinical applications of the heparin-protamine titration test.

TABLE V. HEPARIN-PROTAMINE TITRATION TEST IN A NORMAL SUBJECT BEFORE AND 15 MINUTES AFTER THE INTRAVENOUS ADMINISTRATION OF 50 MG. HEPARIN (SUBJECT A. B., MALE, 70 KG.)

A. BEFORE HEPARIN

PROTAMINE TITRATION		HEPARIN TITRATION	
P*	TIME	H†	TIME
0	22.7	0	23.2
10	26.0	10	36.3
15	28.6	20	71.3
20	31.7	30	210.0
30	34.9		
40	34.7		
50	37.9		

HEPARIN-PROTAMINE TITRATION TEST

H	P	TIME	H	P	TIME	H	P	TIME
0	0	23.5	0	0	23.3	0	0	23.5
10	0	41.4	20	0	78.0	30	0	210.0
10	10	29.3	20	10	57.9	30	10	138.4
10	15	**27.5**	20	20	35.0	30	20	73.7
10	20	29.4	20	30	**26.5**	30	30	46.2
10	25	31.5	20	35	27.4	30	40	29.2
10	30	35.0	20	40	30.2	30	45	**28.8**
10	40	44.4	20	50	34.0	30	50	29.6
10	50	45.2				30	60	34.8
Ratio	1.5:1			1.5:1			1.5:1	

B. 15 MINUTES AFTER 50 MG. HEPARIN INTRAVENOUSLY

PROTAMINE TITRATION	
P	TIME
0	37.3
10	25.9
15	**24.1**
20	25.4
30	29.4
40	33.4
50	35.7

HEPARIN-PROTAMINE TITRATION TEST

H	P	TIME	H	P	TIME	H	P	TIME
0	0	37.7	0	0	38.8	0	0	38.4
10	0	245.0	20	0	1170.0	30	0	1440.0
10	10	95.0	20	10	900.0	30	10	----
10	15	40.9	20	20	267.0	30	20	332.2
10	20	38.1	20	30	48.0	30	30	140.2
10	25	27.8	20	35	31.0	30	40	92.9
10	30	**27.0**	20	40	28.4	30	45	35.1
10	40	27.9	20	45	**27.5**	30	50	31.7
10	45	30.8	20	50	28.7	30	55	27.7
10	50	33.0		60	33.1	30	60	**26.7**
10				70	33.3	30	70	28.6
Ratio	3.0:1			2.25:1			2.0:1	

*P, Concentration of protamine, gamma/ml.
†H, Concentration of heparin, gamma/ml.

Demonstration of Heparinemia in Patients With Hemorrhagic States.—In a variety of clinical conditions it is now suspected that heparinemia or a reduced tolerance to heparin is responsible to some extent for the hemorrhagic manifestations that occur. These conditions include some patients with leucemia, some patients with thrombocytopenic purpura and other blood dyscrasias, some patients who have received nitrogen mustard therapy, some patients and some dogs and swine after total body exposure to ionizing radiations. The evidence for this suspicion in these conditions is as follows.

(a) Reduction of bleeding time, and/or clotting time, and/or cessation of spontaneous bleeding after administration of antiheparin agents: toluidine blue and protamine sulfate.

(b) Abnormal whole blood protamine-heparin titrations.

(c) Abnormal oxalated plasma heparin-protamine titration tests.

A detailed report of a group of patients of this sort in whom abnormal heparin-protamine titration tests were found is in preparation.

We wish to report here two examples of patients of this type in whom the oxalated plasma test returned to normal after effective therapeutic doses of protamine sulfate. Tables VI and VII present the findings and show in each case an abnormal protamine:heparin ratio before treatment, and a ratio of 1.5:1 after treatment.

TABLE VI. HEPARIN-PROTAMINE TITRATION TESTS IN A PATIENT WITH INTRAPERITONEAL HEMORRHAGE DUE TO IDIOPATHIC THROMBOCYTOPENIC PURPURA, BEFORE AND 4 HOURS AFTER THE INTRAVENOUS ADMINISTRATION OF PROTAMINE SULFATE, 2 MG./KG.; THERE WAS SYMPTOMATIC IMPROVEMENT AFTER THIS THERAPY (SUBJECT H. C., FEMALE)

CONCENTRATION USED IN TEST (GAMMA/ML.)		CLOTTING TIME (SEC.)	
HEPARIN	PROTAMINE	BEFORE THERAPY	AFTER THERAPY
0	0	22.5	20.8
20	0	58.7	28.9
20	10	42.0	27.0
20	20	33.0	23.7
20	30	26.5	**21.8**
20	40	**25.6**	23.7
20	50	31.0	27.2
20	60	—	28.2
Ratio		2.0:1	1.5:1

In one case, Subject H. C., the amount of endogenous heparin was estimated as 6.6 gamma per milliliter, and in the other, Subject A. F., it was 3.3 gamma per milliliter. That is, 10 and 5 gamma per milliliter respectively of additional protamine sulfate were required in the test.

TABLE VII. HEPARIN-PROTAMINE TITRATION TESTS IN A PATIENT WITH GINGIVAL BLEEDING DUE TO AN UNDIAGNOSED HEMORRHAGIC STATE, POST SPLENECTOMY, BEFORE AND 24 HOURS AFTER INTRAVENOUS ADMINISTRATION OF PROTAMINE SULFATE, 2 MG./KG.; THERE WAS CESSATION OF BLEEDING SHORTLY AFTER THE DRUG WAS GIVEN (SUBJECT A. F., MALE)

CONCENTRATION USED IN TEST (GAMMA/ML.)		CLOTTING TIME (SEC.)	
HEPARIN	PROTAMINE	BEFORE THERAPY	AFTER THERAPY
0	0	24.9	20.7
10	0	40.8	30.2
10	10	29.2	24.0
10	15	29.0	**22.5**
10	20	**28.3**	25.7
10	30	34.4	29.3
10	40	37.8	33.1
	50	38.7	33.6
Ratio		2.0:1	1.5:1

Quantitative Determination of the Activity of Heparin in Pharmaceutical Preparations.—In the heparin-protamine titration test, as described, the heparin and protamine are the constants, and the oxalated plasma is the variable. By using known normal oxalated plasma, and known concentrations of protamine sulfate, it is possible to assay the heparin content of biologic material, or heparin-containing preparations of any sort. This test may be used, for example, to determine the potency of dried, powdered heparin, obtained at various stages

in the production of commercial heparin. A simple technique for this is as follows. Working standard solutions of 1,000 and 2,000 gamma per milliliter of the unknown material are prepared. Test solutions, with concentrations of 5, 10, 20, 30, 40, 50, and 60 gamma per milliliter respectively, are made up. Each of these dilute solutions is tested for its anticoagulant effect on the clotting time of normal oxalated plasma as already described. The concentration which doubles or trebles the clotting time is the one most suitable for use in the heparin-protamine titration test. Using the dilute solutions of protamine sulfate, the concentration is found which causes the most rapid clotting time. This concentration divided by 1.5 (the critical ratio) gives the concentration of heparin in the solution used in terms of the standard heparin: 110 units per milligram.

DISCUSSION

The fact that protamine sulfate and toluidine blue, known antiheparins, have a favorable effect on certain patients with hemorrhagic states is of great clinical interest. The possibility that heparinemia may be responsible for some hemorrhagic states is of considerable theoretical importance, since it necessitates a revision of contemporary views concerning blood coagulation. The test which has been described has been useful, in our hands, in clinical practice. It has permitted us to detect the patients in whom therapy with the antiheparins is most likely to be effective. With few exceptions, patients have been benefited by the use of protamine sulfate if the heparin-protamine titration test is abnormal. Likewise, with few exceptions, there has been little apparent value from protamine sulfate therapy when the test gives normal results. In this respect, our experience is comparable to that of Allen and co-workers who used a whole blood protamine titration test. Comparisons of these two techniques have been made and will be reported in another paper. The agreement is remarkably good in most instances. The oxalated plasma test has the advantage that it can be performed at one's convenience, and at a distance from the patient. It is also possible to repeat the titration several times on one sample of blood.

We have found that it is possible to control therapy with protamine sulfate by using this test. Excessive or inadequate dosage is readily observed and the treatment schedule can be adjusted daily on the basis of the findings.

The physiologic basis for the phenomenon reported is not at all clear. Attempts have been made to construct a hypothesis which would explain the findings. The simplest hypothesis cannot be proved at present. It is as follows: (1) Heparin is present in small amounts in normal blood and may contribute to its fluidity. The amount required for this action is too small to be detected by any simple method. Variations in clotting due to variations of normal heparin content may represent the normal range of clotting time as usually determined. (2) Heparin is "consumed" or used at a definite rate. It is conceivable that excess production may be so closely matched by excess consumption that little change may be detectable in the blood, because of the wide range of variation in clotting time, and because of the action of other factors. (3) Excessive amounts of heparin in blood or plasma may become evident when an additional amount of heparin is added in vitro. Thus, the addition of 10 gamma of heparin per

milliliter of oxalated plasma increases clotting time by 10 seconds; another 10 gamma increases it by 28 seconds more; and a third increment of 10 gamma increases it by 90 seconds more. Under these circumstances it is likely that a given increment of heparin, added in vitro, will have a greater effect on blood containing say 5 gamma per milliliter than on blood containing 1 gamma per milliliter. We might not be able to discriminate between the two bloods before heparin was added, since our curve (Fig. 2) shows that these amounts alone would give clotting times of 26 and 23 seconds respectively. (4) It seems reasonable to suppose that a person whose endogenous heparin production is five or more times the normal might have some evidence of a hemorrhagic tendency.

This type of explanation is obviously a simplification, since it disregards all the other elements of the clotting mechanism. Regardless of theoretical consideration it is certain that the studies of Allen and his colleagues have emphasized an aspect of blood coagulation which has been largely neglected. The demonstration that circulating anticoagulants may be responsible for some hemorrhagic states is important. The exact role of such substances in disease states remains to be determined.

The heparin-protamine titration test which we have described represents another effort to study abnormal blood clotting in a manner which can be controlled to some extent. It is of interest that it is possible to utilize this same technique for the assay of heparin-containing materials of any sort. It is not an absolute test, since it is necessary to work always with a reference standard. In this instance we have used the Connaught Laboratories' heparin as the standard. The consistency of the relationship between protamine sulfate and heparin is so great, however, that it should be possible to use the reaction described as a primary assay technique.

SUMMARY

1. A method has been described for the detection of heparinemia.

2. This method is a heparin-protamine titration test using a modified one-stage prothrombin time test as the indicator.

3. The application of this test to clinical medicine is discussed.

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ESTIMATION OF SERUM GAMMA GLOBULIN CONCENTRATION BY TURBIDIMETRY

J. DE LA HUERGA, M.D.,* AND HANS POPPER, M.D., PH.D.
CHICAGO, ILL.

IN RECENT years, flocculation and turbidity tests have been found useful in the differential diagnosis of hepatobiliary disorders. Since elevation of the serum gamma globulins is known to be an important factor in these tests, it was attempted to develop a turbidity test more specific for this serum fraction.

The gamma globulin of serum is defined by its electrophoretic mobility. Recently, several chemical methods of determining this fraction using salt precipitation have been described.[1, 2, 3, 4] A turbidimetric method of gamma globulin determination, based upon the chemical method of Wolfson and coworkers[1] is herein described. It follows in principle the procedure of the thymol turbidity test.[5] The influence of variations in temperature, time of development, shaking, and pH—factors known to affect flocculation tests—is discussed.

In subsequent papers, the results of determinations using this procedure are compared with those obtained from electrophoretic partition and the zinc sulfate turbidity test.[6] The applications of these findings to various clinical disorders will be discussed.

METHOD

Reagent.—One hundred eighty-nine grams of ammonium sulfate and 29.3 Gm. of sodium chloride are dissolved in distilled water and the volume of the solution is made to 1 liter. The sodium chloride concentration is lower than that used in the original reagent of Wolfson and associates.[1] However, a personal communication from Dr. C. Cohn advised the use of a 25 per cent lower concentration for the chemical method and it was therefore proportionally reduced in the turbidimetric method.

Procedure.—With a micropipette, 0.10 ml. of serum is transferred to a test tube or colorimeter cuvette and 5 ml.† of the reagent are added. After allowing the mixture to stand for thirty minutes, the test tube or cuvette is inverted twice without shaking and the turbidity is read in a spectrophotometer or photometer using a wave length of 650 mμ or a red filter with a similar transmission, respectively. The reagent serves as a blank. The per cent of transmission is interpolated on the standard curve used for the thymol turbidity test. The results are expressed either in units similar to the thymol turbidity test or, better, in grams gamma globulins per 100 ml. serum. If the curve of Shank and Hoagland[7] (based on a precipitation of a 0.0962 normal barium chloride solution by 0.2 normal sulfuric acid) is used, the following formula permits conversion of units into grams gamma globulins per 100 ml. independent of the instrument applied.

$$\text{Gamma globulins, grams per 100 ml.} = \frac{\text{Units}}{5.8} + 0.02\ (20 - \text{units})$$

From the Hektoen Institute for Medical Research and the Departments of Pathology of Cook County Hospital and Northwestern University Medical School.
Supported by a grant from the Dr. Jerome D. Solomon Memorial Research Foundation.
Received for publication, Nov. 18, 1949.

*Solomon Fellow.

†In contrast to thymol turbidity and zinc sulfate turbidity, 5 ml. instead of 6 ml. are used for dilution in order to obtain a higher concentration of the turbidity in normal instances and to permit the use of widely available volumetric pipettes. If the cuvette of the instrument used requires more fluid than 5 ml., higher aliquots such as 0.2 and 10 ml. are recommended.

To obtain a reproducible barium sulfate calibration curve, four different aliquots each of the barium chloride solution representing 20 units and 8 units are read and the means of the two levels are plotted on semilogarithmic paper. For the copper sulfate calibration curve of Ducci,[8] the following formula applies if a monochromatic spectrophotometer is used.

$$\text{Gamma globulins, grams per 100 ml.} = \frac{\text{Units}}{24.4} + 0.004\ (100 - \text{units})$$

However, since the light absorption of copper sulfate solutions is more influenced by the wave length than that of barium sulfate, the formula does not apply for a filter photometer (which may also record different thymol turbidity units with the same reagent in view of the wider band of wave length). In this instance or if a basically different curve or visible standards are used or if an additional check is desired, the curve in use can be calibrated by determining the gamma globulin concentration of several serums simultaneously by the chemical and by the turbidmetric method and by plotting the units against the concentration in grams per 100 ml. of serum.

RESULTS

Correlation Between the Turbidimetric and the Chemical Method.—The results of turbidimetric readings were compared with the amount obtained by the chemical method of Wolfson and co-workers[1] in 150 normal serums as well as in serums from patients with different diseases (Fig. 1). The points form a straight line which reaches the base line at 0.4 gram per 100 milliliters. This deviation from a line which goes through the origin is taken into account in the conversion formula given (Fig. 2).

Characteristics of the Precipitate.—

(a) Duplication of the Results: In more than 100 serums in which up to five duplicate determinations were performed, the difference between the parallel samples never exceeded 0.1 gram per 100 milliliters.

(b) Stability of the Precipitate: The precipitate present after inverting the tube revealed under the microscope particles of approximately equal size. It remained well distributed for at least ten minutes.

(c) Influence of Time: The turbidity readings developed irregularly with time. Turbidity increased rapidly within the first fifteen minutes, then became fairly constant for more than one hour. The readings after twenty-four hours exceeded those after thirty minutes by 10 to 15 per cent. This increase in turbidity is not due to an increased amount of precipitated protein since chemically the amount of precipitate after ten minutes was identical to that after forty-eight hours. The increase in turbidity is probably the result of hydration of the precipitate. The selected time interval of thirty minutes appears optimal.

(d) Influence of Temperature: When the reaction was performed in the ice box, about 30 per cent less turbidity was noted; while when it was performed at 37° C., a 30 per cent higher turbidity was observed than at room temperature. These differences are also probably due to alterations in hydration. Variation in temperature between 20 and 30° C. produced differences which did not exceed 1 per cent per degree centigrate. For very exact determinations, it may be advantageous to perform the test at 25° C. In serums previously kept at 56° C. for one-half hour, the precipitate determined chemically or turbidimetrically showed an increase of up to 50 per cent.

(c) Acidity of the Reagent: The reagent used has a pH of approximately 5.5 due to the acidity of the ammonium sulfate. Neutralization of the reagent by ammonium hydroxide increased the amount of protein precipitated by about 20 per cent with the chemical and turbidimetric methods. Jager and Nickerson's method[2] which uses a neutralized solution of ammonium sulfate

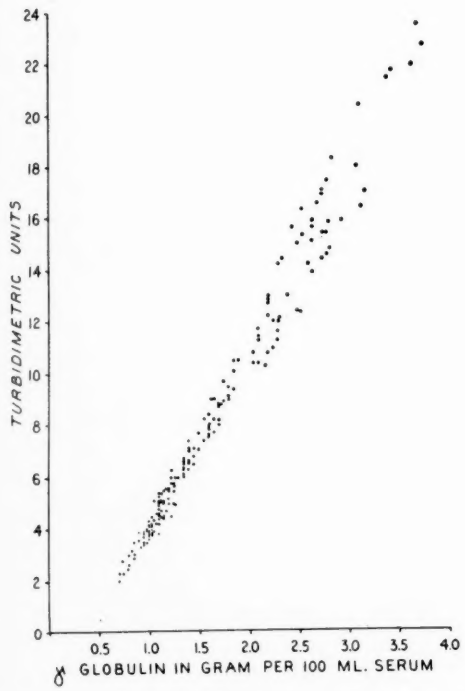

Fig. 1.

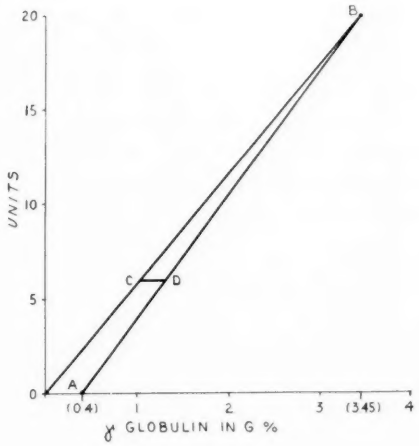

Fig. 2.

Fig. 1.—Correlation between the units determined turbidimetrically and grams of gamma globulin per 100 ml. of serum as determined chemically.

Fig. 2.—Calculation of the formula for conversion of units into grams of gamma globulins per 100 ml. of serum by use of the calibration curve of Shank and Hoagland.[7] If the line $0B$ were to represent the plot line between turbidimetric units and grams per 100 ml., the value for grams per cent would be $U \times \frac{3.45}{20} = \frac{U}{5.8}$ (U represents the turbidimetric units) inasmuch as 20 turbidimetric units represent 3.45 grams per 100 milliliters. Since the actual line (AB) reaches the abscissa at 0.4 gram per 100 ml., the difference (CD) can be calculated as follows:

$$\frac{CD}{20 - U} = \frac{0A}{20}$$

$$CD = \frac{(20-U) \times 0.4}{20} = 0.02 \quad (20-U)$$

The corrected formula is then $\frac{U}{5.8} + 0.02 \times (20-U)$.

of slightly lower concentration gives results identical with those of the method of Wolfson and associates.[1] Since the results of both methods are supposed to check fairly well with electrophoretic determinations and with the generally accepted gamma globulin levels, neutralization of the reagent was not used in this study.

Recovery Experiments.—The degree of recovery in serum or saline was determined by simultaneous chemical and turbidimetric measurements following the addition of either commercial immune gamma globulins or a preparation obtained by special fractionation.* In three experiments in which these gamma globulin preparations were added to saline solutions to produce a final concentration of from 1.2 to 4 grams per 100 ml., the recovery by both methods varied from 74 to 80 per cent. Gamma globulin was added to two serums to raise their gamma globulin concentration by 1.2 and 1.3 grams per 100 milliliters. In these instances, 83 to 87 per cent of the added globulin was recovered. As in the previous series, turbidimetric and chemical procedures gave the same results.

Salt-free human albumin† was added to saline solutions containing 2.50, 2.30, 1.30, 1.20, and 0.9 grams of gamma globulins per 100 ml. to produce an albumin concentration of 1.25, 2.5, 5.0, and 7.5 grams per 100 ml. without influencing the turbidimetric readings. Similarly, addition of albumin solution to seven serums to raise their albumin concentration by 1, 2, 3, 4, and 5 grams per 100 ml. did not influence their turbidimetric readings.

Procedure for Serums With High Gamma Globulin Content.—If, after adding the reagent to the serum, a very high turbidity appears (over 3 grams per 100 ml.), another 5 ml of reagent are added to the mixture and the results obtained are multiplied by two.

Correction for Lipemic and Jaundiced Serums.—Only marked lipemia or excess jaundice increased the turbidimetric readings; e.g., when the serum bilirubin is 50 mg. per 100 ml. the error is 0.1 gram per 100 milliliters. However, this source of error can be excluded by the following procedure: after the reading of the turbidity, 2.5 ml. of distilled water are added to the serum-reagent mixture and the colorimeter tube is inverted. This dissolves the entire gamma globulin turbidity. The remaining light absorption due to the yellow color or lipid turbidity is recorded using the same wave length or filter. The second reading is interpolated on the standard curve, the result multiplied by 1.5, and then subtracted from the original value.

Slight hemolysis does not interfere with the method since it can also be corrected for as described.

Influence of Lipid Intake.—The gamma globulin turbidity of four patients was determined before and three and six hours after the intake of 50 grams of butter. No differences in the turbidity were noted when the proper correction for lipemia was made.

Gamma Globulin Values.—

(a) Controls: In fifty-two normal persons (interns, nurses, technicians), the gamma globulin, measured turbidimetrically, varied from 0.7 to 1.25 grams per 100 ml. of serum (Fig. 3). The mean was 1.03 grams per 100 ml. and only in one instance did the concentration slightly exceed 1.25 grams. This can, therefore, be considered the borderline of normal values.

*Generously supplied by Dr. H. F. Deutsch of the Department of Physiological Chemistry, University of Wisconsin, Madison, Wis.

†Obtained through the Red Cross.

(b) Blood Donors: Serums of 100 donors of the blood bank of Cook County Hospital were examined chemically and turbidimetrically. The mean of these samples (Fig. 3) was 1.20 grams of gamma globulin per 100 ml. with a standard deviation of ±0.24. Of these, 23 per cent showed values above 1.25 grams per 100 ml., extending up to 1.8 grams per 100 milliliters.

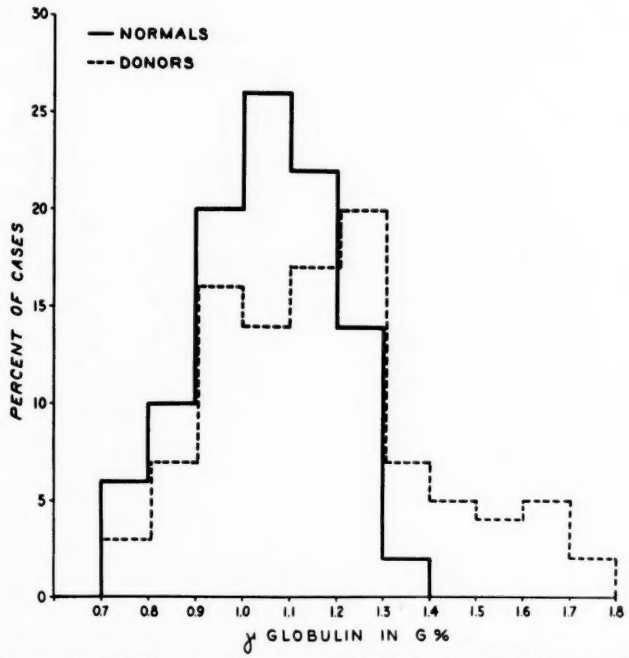

Fig. 3.—Percentage distribution of the gamma globulin concentration expressed in grams per 100 ml. of serum in 52 normal persons and 100 blood donors.

DISCUSSION

Presentation of a turbidimetric test for serum gamma globulins requires justification for two reason: first, because it increases the larger number of flocculation and turbidity tests already used in the differential diagnosis of hepatobiliary diseases, and second, it is to replace the chemical determination based on salt partition and biuret reaction.

Most of the flocculation tests known are based on the interplay of several factors. The cephalin flocculation,[9] the colloidal gold,[9, 10] colloidal red,[11] and the Takata-Ara[12] tests are based primarily on elevation of gamma globulins and reduction or alteration of albumin. In the thymol turbidity test,[13, 14] in addition, lipids and lipoproteins bound to beta globulins play a role. Predominance of one or the other of these factors explains the differences in the results of the various tests in individual instances.[15]

The proposed test is designed to mirror only the gamma globulin concentration. However, the gamma globulin fraction as such is not too clearly defined; it consists of several protein fractions even on the basis of electrophoretic mobility.[16]

The salt partition applied in this test is said to precipitate about 90 per cent of the gamma globulins and only a small fraction of the other serum proteins. However, complete and specific salt precipitation is not possible in one step.[17] This explains why the recovery chemically and turbidimetrically is rather poor although the results of this method of precipitation correlate well with those of electrophoresis.[18] In this sense, the term gamma globulin turbidity test is proposed without claiming complete identity with any single protein fraction. Since variations in serum gamma globulins occur in a large number of diseases and appear characteristic for certain phases, application of this method may be fruitful, especially if it is used serially. The simpler turbidimetric method appears as reproducible as the chemical method in view of the proved stability of the turbidity if the conditions are standardized. Albumin and lipids which influence other flocculation tests do not alter the results of the method presented.

The upper limit of the normal in serum is 1.25 grams per 100 ml., this figure being in good agreement with results obtained by electrophoretic[17] and chemical methods.[1, 2] It is interesting that blood donors whose health is taken for granted but who have not been clinically examined reveal an appreciable incidence of elevated gamma globulins. The cause of this elevation is being investigated.

SUMMARY

1. A turbidity test is proposed which measures the gamma globulin concentration of the serum, adapting the procedure used in the thymol turbidity test. The method is based upon the salt precipitation used in the chemical method of Wolfson and co-workers,[1] with which its results are identical. Results are recorded in grams of gamma globulins per 100 ml. of serum or may be expressed in the same units used in the thymol turbidity.

2. The characteristics of the turbidity are described. It is independent of the albumin and lipid concentration of the serum. Recovery of added gamma globulins is the same as in the chemical method. The values obtained do not mirror the gamma globulin concentration exactly, but approximate it as closely as the chemical method.

3. In normal individuals, the values ranged between 0.7 and 1.25 grams per 100 ml. of serum. In blood donors, taken at random, higher gamma globulin concentrations were found occasionally.

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COMPARISON OF THE RESULTS OF GAMMA GLOBULIN AND ZINC SULFATE TURBIDITY TESTS WITH ELECTROPHORETIC DETERMINATION OF GAMMA GLOBULINS

J. DE LA HUERGA, M.D.,* AND HANS POPPER, M.D., PH.D., CHICAGO, ILL., AND MURRAY FRANKLIN, M.D., AND J. I. ROUTH, PH.D., IOWA CITY, IOWA

IN THE preceding paper, a method was described for the turbidimetric estimation of the gamma globulin concentration in serum.[1] Since gamma globulin is defined as a serum protein fraction with a characteristic electrophoretic motility, it appears pertinent to compare the results of the gamma globulin turbidity test with those of electrophoretic partition. In recent years, the zinc sulfate turbidity test was devised to estimate alterations of serum gamma globulin concentrations.[2, 3] Therefore, the results of the latter test were also compared with electrophoretic analyses and at the same time the factors influencing the results of the zinc sulfate turbidity were investigated in order to provide a basis for comparison of the two turbidity tests.

MATERIAL AND METHODS

From normal human subjects and patients with various disorders (especially liver disease and myeloma), 156 serum or oxalated plasma specimens were subjected to electrophoresis in the Tiselius apparatus. Prior to electrophoresis the specimens were diluted with buffer to a protein concentration of 1.5 to 2.0 per cent and dialyzed at 2 to 6° C. for three days with daily change of buffer. Barbiturate buffer with a pH of 8.6 and an ionic strength of 0.1 was used and electrophoresis was carried out at 0.8° C. in the 11 c.c. analytical cell for 120 minutes at a current of 25 milliamperes. The patterns were enlarged two and one-half times and the tracings analyzed for area distribution with a planimeter. The values reported are those obtained from the descending boundaries. In the same serum, or in the case of plasma, in serum obtained at the same time as the plasma, the gamma globulin turbidity as well as the zinc sulfate turbidity were determined using the calibration curve of Shank and Hoagland[4] based on 0.0962 normal barium chloride. The results of the zinc sulfate turbidity were given either as units or in grams per cent gamma globulin calculated according to the conversion formula of Kunkel and co-workers.[3] The gamma globulin concentration determined by electrophoresis was plotted against that obtained by the turbidimetric methods, and the significance of the correlation was tested by Pearson's product-moment correlation coefficient.

$$r_{xy} = \frac{\Sigma_{(xy)}}{N\sigma_x\sigma_y}$$

where x and y are the deviations of the observed values from the respective means. N is the total number of cases and σ_x and σ_y are the standard deviations of the respective means.

From the Hektoen Institute for Medical Research and the Departments of Pathology of Cook County Hospital and Northwestern University Medical School, Chicago, Ill., and the Departments of Internal Medicine and Biochemistry, College of Medicine, State University of Iowa, Iowa City, Iowa.

Supported by a grant from the Dr. Jerome D. Solomon Memorial Research Foundation.

Received for publication, Nov. 18, 1949.

*Solomon Fellow.

An r of 1.0 represents perfect correlation; whereas an r of 0 indicates lack of correlation. The standard error of estimate of the turbidmetric determination in both instances was tested according to the formula:

$$S_y = \sigma_y \sqrt{1 - r^2}$$

where σ_y = standard deviation as before and r is the correlation coefficient.

The mean (with the standad deviation) of the difference of the results of the turbidimetric and the electrophoretic determinations of the same samples, expressed in per cent of the electrophoretically determined concentration, was calculated on the entire sample as well as in three groups divided according to the electrophoretically determined gamma globulin concentration.

In addition, 10 c.c. from each of three serums were diluted with the ammonium sulfate-sodium chloride solution used for the gamma globulin turbidity test.[1, 5] The precipitate formed was collected and redissolved in saline solution. The supernatant fluid was then saturated with ammonium sulfate to precipitate the remaining protein fractions not precipitated by the first dilution with ammonium sulfate. The collected second protein fraction was also redissolved in saline solution and both fractions were submitted to electrophoretic analysis after dialysis.

RESULTS

1. Comparison Between Electrophoretic and Turbidimetric Determinations.—The gamma globulin concentration determined by electrophoresis correlated well with the one estimated by turbidimetry in the same serum (Fig. 1). The r is high and the standard error of estimate is 0.34 grams per 100 milliliters. The results of the zinc sulfate turbidity agreed less well with the electrophoretic readings for gamma globulin in the same serum, if the latter was expressed in grams per cent gamma globulin (Fig. 2) or in units (Fig. 3). The r value was considerably lower in both instances than in the case of the gamma globulin turbidity and the standard error of estimate was 0.44 gram per cent or 5.8 units. The correlation of the electrophoretically determined gamma globulins with the zinc sulfate turbidity expressed in units was, in general, slightly better than with the results expressed in grams per 100 ml. serum. This was especially true in the ranges above normal where the gram values of the zinc sulfate turbidity showed a tendency to be lower than would be expected from electrophoretic readings. In the normal ranges, however, the unit values appeared much lower. In this instance (Fig. 3), a straight line through the points reached the zero line of the zinc sulfate turbidity at around 0.5 gram electrophoretically determined gamma globulin per 100 milliliters.

2. Percentage Error of the Gamma Globulin and Zinc Sulfate Turbidity in Comparison With Electrophoretic Determinations.—The mean of the percentage difference of gamma globulin concentrations determined by electrophoresis and by the ammonium sulfate-sodium chloride turbidity test was not quite 15 per cent, if the whole sample was considered (Table I). If the cases were grouped according to the gamma globulin concentration, the highest mean percentage difference was found in the low region of the normal range. It was the lowest with moderate elevation of the gamma globulin concentration and slightly higher with elevation of the gamma globulin concentration above

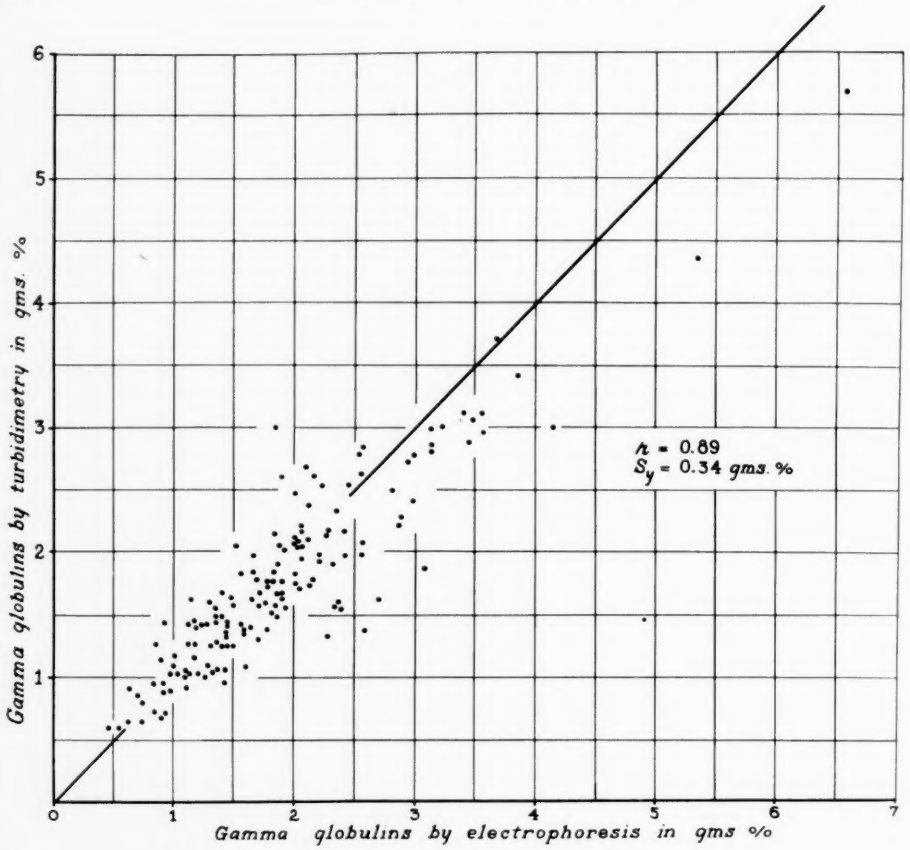

Fig. 1.—Plot of the gamma globulin concentration determined electrophoretically versus the one determined turbidimetrically in normal and pathologic serums.

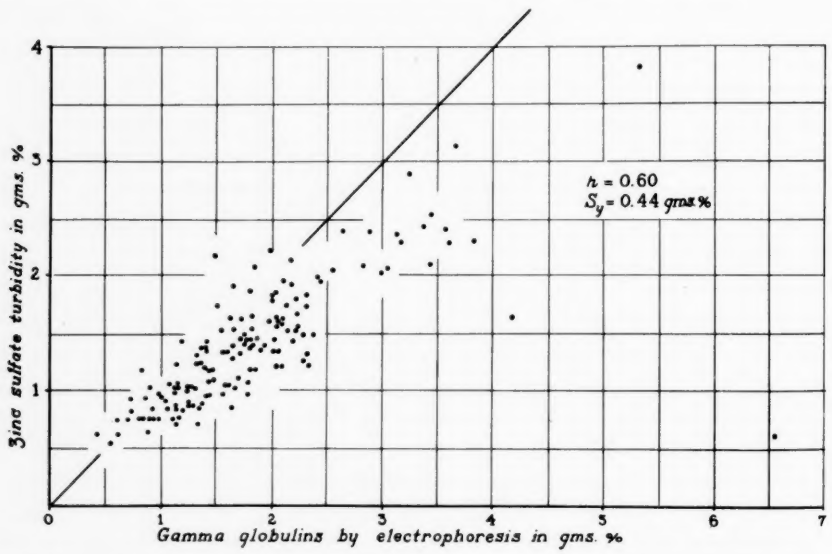

Fig. 2.—Plot of the gamma globulin concentration determined electrophoretically versus the results of the zinc sulfate turbidity expressed in grams gamma globulin per 100 ml. in normal and pathologic serums.

2.50 grams per 100 c.c. serum. In the same 156 cases, the mean percentage difference between electrophoretic reading for gamma globulin and zinc sulfate turbidity was 24.5 per cent with a standard deviation of ±14.6

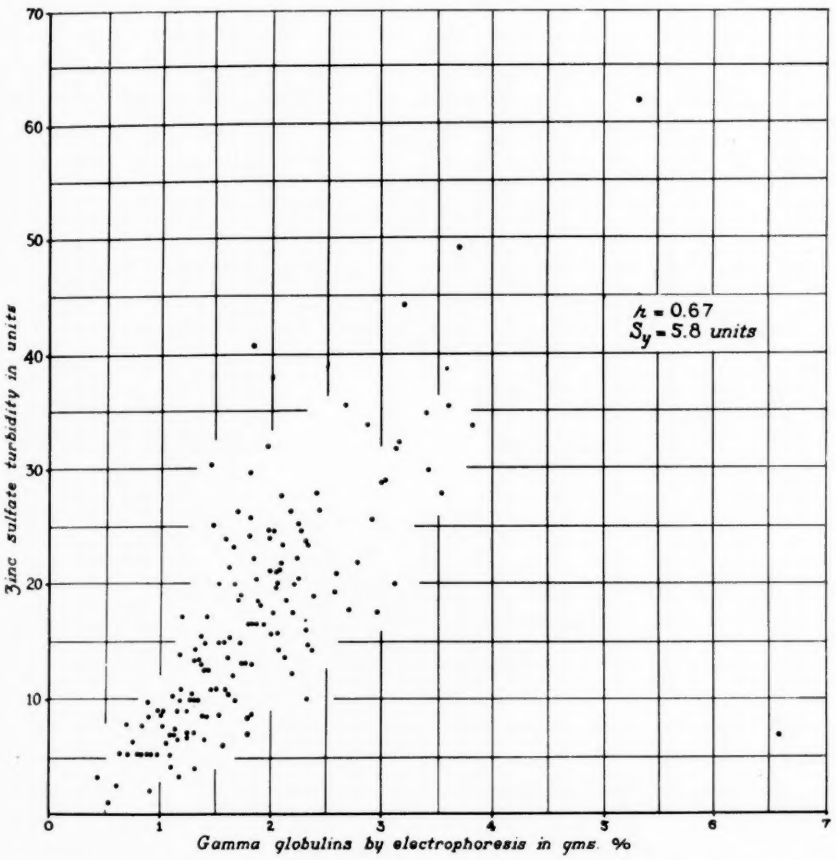

Fig. 3.—Plot of the gamma globulin concentration determined electrophoretically versus the results of the zinc sulfate turbidity expressed in units in normal and pathologic serums.

3. Electrophoretic Analysis of the Precipitate Produced by the Ammonium Sulfate-Sodium Chloride Solution.—The precipitate formed consisted of approximately three-fourths of gamma globulin, apparently of the character of the gamma$_2$ fraction of Deutsch.[6] Approximately one-sixth is formed by a

TABLE I. PERCENTAGE DIFFERENCE BETWEEN THE GAMMA GLOBULIN CONCENTRATION DETERMINED ELECTROPHORETICALLY AND TURBIDIMETRICALLY, THE FORMER BEING TAKEN AS STANDARD; THE CASES ARE GROUPED ACCORDING TO THE GAMMA GLOBULIN LEVEL DETERMINED ELECTROPHORETICALLY

CONCENTRATION OF GAMMA GLOBULIN IN GM. % DETERMINED ELECTROPHORETICALLY	NUMBER OF CASES	MEAN PERCENTAGE DIFFERENCE	STANDARD DEVIATION
0-1.25	34	17.1	±14.0
1.26-2.50	96	13.5	±10.2
2.51 and above	26	14.3	± 9.7
Total	156	14.6	±11.1

protein fraction which consists partly of gamma$_1$ globulin and partly of beta globulin. It could not be clearly identified by electrophoresis since its mobility lies between that of beta and gamma globulin. Only a relatively small fraction (less than 10 per cent) was composed of the other serum protein fractions. In contrast, in the supernatant fluid, only little gamma globulin of gamma$_1$ as well as of gamma$_2$ character remained unprecipitated (Table II and Fig. 4). However, these relatively small percentage concentrations are significant in view of the fact that the greater part of the proteins remained in the supernatant fluid.

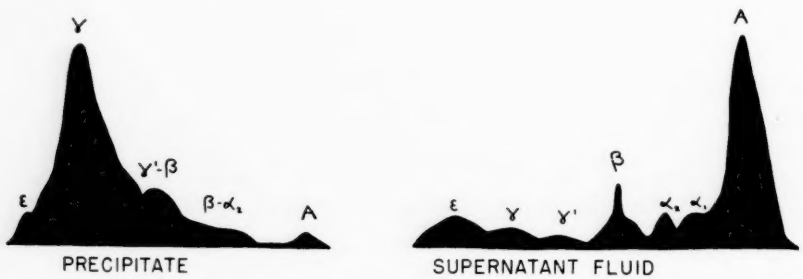

Fig. 4.—Electrophoretic tracing (descending boundaries of precipitate and supernatant fluid of Case CB).

4. Studies as to the Mechanism of the Zinc Sulfate Turbidity.—

(a) Effect of Addition of Albumin: Addition of salt-poor human serum albumin to gamma globulin solutions to produce a concentration of 1 gram per 100 ml. increased the zinc sulfate turbidity 5 to 6 units. A further increase of the albumin concentration reduced the zinc sulfate turbidity considerably, even below the turbidity of the pure gamma globulin solutions (Fig. 5). Increasing amounts of albumin added to serums depressed the zinc sulfate turbidity in almost a straight-line fashion (Fig. 6). Increasing the albumin concentration of ten normal and pathologic serums by 1.5 grams per 100 ml. depressed the zinc sulfate turbidity between 20 and 50 per cent. In general, the percentage of the depression was lower with high zinc sulfate turbidity. In both series the gamma globulin turbidity was not influenced by the albumin addition.

TABLE II. RELATIVE PROPORTION (IN PER CENT) OF THE PROTEIN FRACTIONS AS DETERMINED ELECTROPHORETICALLY IN THE PRECIPITATE PRODUCED BY DILUTION OF SERUM WITH AMMONIUM SULFATE-SODIUM CHLORIDE SOLUTION AS WELL AS IN THE SUPERNATANT FLUID

PATIENT	SERUM GAMMA GLOBULIN IN GM. % BY ELECTROPHORESIS	PRECIPITATE				SUPERNATANT FLUID					
		ALBUMIN	GLOBULIN			ALBUMIN	GLOBULIN				
			α_2–β	β–γ_1	γ		α_1	α_2	β	γ_1	γ
J. D.	2.12	3.8	3.0	19.4	73.8	56.3	4.9	3.8	22.8	3.5	8.7
C. B.	2.55	2.0	8.1	14.1	75.8	66.0	7.5	5.4	11.6	3.0	6.5
J. H.	2.17	1.5	2.7	16.8	79.0	50.4	5.5	12.6	8.9	6.2	16.4

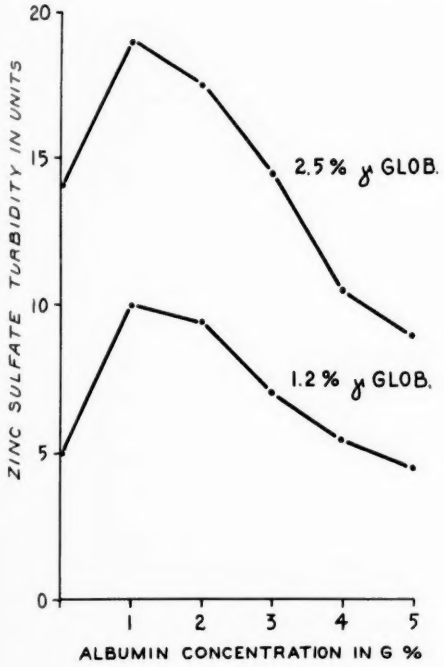

Fig. 5.

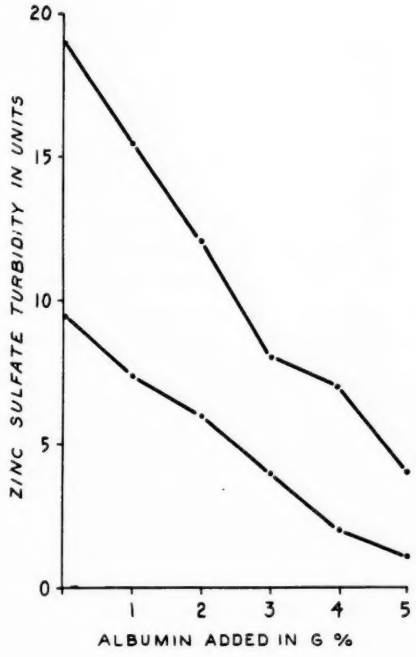

Fig. 6.

Fig. 5.—Influence of addition of increasing amounts of albumin on the zinc sulfate turbidity in saline solutions containing 2.5 and 1.2 grams of gamma globulin for 100 milliliters.

Fig. 6.—Influence of addition of increasing amounts of albumin on the zinc sulfate turbidity in two serums.

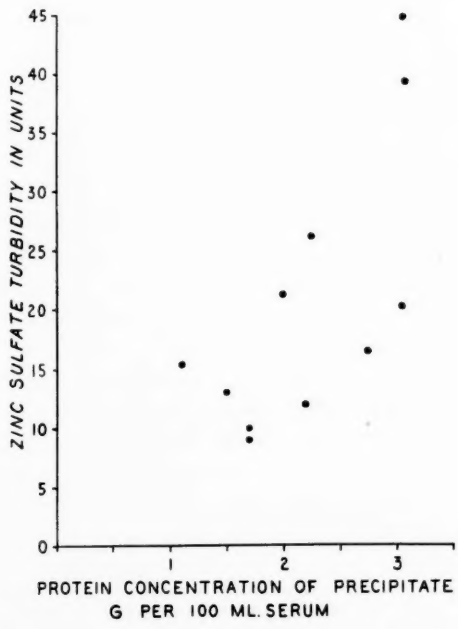

Fig. 7.—Plot of zinc sulfate turbidity expressed in units against the protein concentration of the precipitate formed by zinc sulfate expressed in grams per 100 ml. of serum.

(b) Relation of Zinc Sulfate Turbidity to the Amount of Protein Precipitated: The precipitate formed by addition of zinc sulfate to serum was centrifuged and its protein concentration revealed some correlation to the turbidity, but it was far less definite than in the case of the gamma globulin turbidity (Fig. 7). In some serums, the turbidity could not be removed completely by centrifugation nor by the addition of 1 drop of half normal sodium hydroxide solution (which completely dissolves the precipitate of pure gamma globulin). Such cases were not included in this series.

DISCUSSION

The correlation between the results of the gamma globulin turbidity test and those of electrophoresis reveals only occasionally significant discrepancies. In the vast majority of cases, the difference between the results of both methods is only slightly greater than one would expect from two basically different methods. The electrophoretic determination of gamma globulin has an error of about ±5 per cent[7] depending upon the resolution of the peaks which, especially in liver disease, are not clear. The reproducibility of the turbidimetric method is probably of equal magnitude. It may possibly be better than that of electrophoresis as was seen in serial studies in which the results of the turbidimetric method followed a straighter line than those of the electrophoretic one.

Breakdown of the cases according to the gamma globulin level reveals the greatest percentage error in the normal range. Here relatively small absolute differences produce significant percentage differences. In the markedly elevated range, the conditions are often abnormal, as in myeloma, where salt precipitation and electrophoresis probably separate entirely different protein fractions.[7] The difference between both methods is the lowest in the range of moderate elevation where this method has the greatest practical significance. The correlation also may be impaired by the difficulty encountered in electrophoretic separation of the gamma globulins in liver diseases.[8]

Therefore, for clinical application and serial studies, the simple turbidimetry may be used in place of the rather elaborate electrophoresis; the latter as standard is obviously still required for the confirmation of crucial points. It should be stressed that the good statistical correlation between both methods does not imply that the same type of proteins are determined by both methods. The turbidimetric method records most of the electrophoretically determined gamma globulins but also some of the other protein fractions, especially one which cannot be differentiated well by electrophoresis from gamma globulin. Conversely, the supernatant fluid still contains some gamma globulin in addition to the bulk of the other proteins. Therefore, the protein fractions other than gamma globulin may quantitatively compensate for the gamma globulin missed in the turbidity test. However, exactly the same situation holds true for any of the salt precipitation methods[7, 9, 10] and especially for the classical partition of albumin and globulin according to Howe.[11]

The comparison with the zinc sulfate turbidity was undertaken because of its popularity and also because of the good diagnostic results obtained with it.[3, 12, 13, 14] Statistically, the zinc sulfate turbidity correlates less well with electrophoretic partition of gamma globulin and the expected error is almost twice that obtained with the ammonium sulfate-sodium chloride reagent. In addition, the degree of correlation is different if the zinc sulfate turbidity is expressed in units instead of in grams per cent. The reasons for the less satisfactory correlation in the case of the zinc sulfate turbidity are apparently the influence of albumin (already demonstrated in Kunkel's first publication[2] and confirmed here), the influence of lipid intake,[13] and finally the poor correlation between the turbidity and the protein content of the precipitate. The latter may be explained by the precipitation of nongamma globulin substances (possibly of lipid character) by the zinc sulfate reagent. For estimation of the gamma globulin concentration, the present test is, therefore, superior to the zinc sulfate turbidity, especially since it is equally simple from the technical point of view. The recent modification of the zinc sulfate turbidity test by Looney[15] avoids sensitivity to added albumin. However, the ghatti gum used lowers the pH of the reagent and even after readjustment of the pH to 7.5, the recoveries of added gamma globulins are poor.

Obviously, it cannot be claimed that the present method necessarily gives the best correlation of all simple turbidity methods with electrophoretic determination of gamma globulin, but the degree of correlation seems to suffice for practical clinical usage.

SUMMARY

The results of the gamma globulin turbidity test were correlated with the electrophoretically determined gamma globulin concentration in serum and plasma in 156 instances, including normal persons and patients suffering from various diseases. There was an average difference of about 15 per cent for the entire sample, the difference being less in the moderate pathologic range. Thus, the gamma globulin turbidity test may be used for simple clinical estimation of the gamma globulin concentration. The zinc sulfate turbidity test does not correlate as well with electrophoresis. In contrast to the gamma globulin turbidity test, the zinc sulfate turbidity is influenced by the albumin concentration of the serum and by lipid intake. In addition, the degree of turbidity does not correspond well with the protein concentration of the precipitate. Despite good statistical correlation, the protein fraction determined turbidimetrically is not identical with the electrophoretic gamma globulin. However, the same difficulty is encountered in any salt precipitation method in clinical use and may not necessarily detract from the practical applicability of the turbidity test.

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A RAPID COLORIMETRIC METHOD FOR THE DETERMINATION OF INULIN IN PLASMA AND URINE

AEME HIGASHI, M.S., AND LAWRENCE PETERS, PH.D.
CLEVELAND, OHIO

A NUMBER of colorimetric methods have been employed for determination of the inulin content of plasma and urine during renal clearance studies involving this substance. In this country most of the published methods are based on the production of a blue color by the interaction of diphenylamine and the acid-hydrolysis products of inulin.[1, 2, 3, 4] Several European investigators have published methods based on the application to inulin determination of Roe's modification of the Seliwanoff reaction for fructose.[5, 6] One of these procedures[5] has been tried in this laboratory with unsatisfactory results. Bacon and Bell[7] determined the fructose content of the blood of fetal sheep by a modification of Roe's method developed by Cole. In this procedure the addition of ferric chloride to the reaction mixture of fructose, resorcinol, and hydrochloric acid resulted in more consistent color development. Bacon and Bell stated that the method had been applied to inulin and on this basis it was given trial in this laboratory. The results are somewhat variable, but were improved greatly by substitution of a trichloracetic acid filtrate for the cadmium hydroxide filtrate described. When extremely rigid temperature control was included in the modification, satisfactory results were obtained consistently. The following is a description of the procedure finally adopted for use in this laboratory in connection with studies of the renal clearance of inulin in dogs.

EXPERIMENTAL

Reagents.—

Trichloracetic Acid: A 10 per cent aqueous solution.

Resorcinol: Reagent grade, a 0.15 per cent solution in 95 per cent ethanol.

HCl-Ferric Chloride: Concentrated HCl (37 per cent $\frac{w}{v}$), containing 7.5 mg. of ferric chloride per liter.

Inulin: The inulin used in this study was the ampuled material supplied by the U. S. Products Company. Prior to use in the preparation of standard solutions it was recrystallized several times from hot water by the addition of 95 per cent ethanol. Stock solutions were prepared by dissolving 100 mg. of inulin in 100 ml. of a saturated aqueous solution of benzoic acid. Solution was facilitated by gentle warming. No solubility difficulties were encountered at this concentration, even though solutions were stored at 5° C.

Analytical Procedure.—

Plasma: Two milliliters of water and 12 ml. of cold trichloracetic acid were added to 1 ml. of dog plasma known to contain between 12 and 20 mg. of inulin per 100 milliliters. The mixture was agitated thoroughly and filtered through No. 1 Whatman filter paper.

From the Department of Pharmacology, School of Medicine, Western Reserve University.

This investigation was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, United States Public Health Service.

Received for publication, Nov. 3, 1949.

Urine: All samples were diluted with water to reduce the concentration of inulin to between 20 and 40 mg. per 100 milliliters. One milliliter of the diluted urine then was diluted with water and trichloracetic acid as described for plasma. Filtration was not necessary except in cases of proteinuria.

Standards: Two and one-half milliliters of water and 12 ml. of trichloracetic acid solution were added to 0.5 ml. of the stock solution of inulin described.

Two milliliter aliquots of the plasma filtrate and of the acid urine solution were used in the color reaction. The aliquots of the standard inulin solution were only 0.5 and 1.0 ml. in volume; hence, these were made up to 2 ml. with 8 per cent trichloracetic acid.

Pyrex test tubes (178 by 22 mm.) were used for the color reaction. Three milliliters of alcoholic solution of resorcinol were added to each aliquot of 2.0 milliliters. The next step consisted of the addition of 3.0 ml. of concentrated HCl, containing ferric chloride as described. The tubes were immersed in a beaker of ice water during the addition of the acid in order to prevent an excessive and variable degree of overheating. On the other hand, excessive cooling was avoided by restricting the time of immersion in ice water to the actual period during which acid was added. A satisfactory procedure consisted of the immersion of three tubes at one time and the rapid addition of acid to them from a 10 ml. serologic pipette, with constant agitation. This relatively crude procedure for stabilization of temperature proved to be adequate and obviated the necessity for treating each tube as a separate unit throughout the entire procedure, as described by Bacon and Bell.[7] The color was then developed by heating the rack of tubes in a water bath at 79 to 79.5° C. for twenty minutes. Since the constancy of the temperature was a critical factor, the water bath was controlled in such a way as to permit attainment of the level of 79 to 79.5° C. within one to three minutes after immersion of the tubes. At the end of the heating period the tubes were cooled rapidly, by immersion in ice water, to a temperature of approximately 20° C. Further cooling resulted in clouding, though this disappeared shortly after return to room temperature. The intensity of the orange-red color, which remained unaltered for four hours, was measured in a Klett-Summerson photoelectric colorimeter with filter No. 54.

Blanks: Some of the color produced obviously resulted from interaction of the combined reagents, since control tubes containing no inulin gave readings of considerable magnitude when read against distilled water as a blank. Most of the work was done with inulin concentrations in the range of 15 to 30 γ per tube. In tubes containing 30 γ of inulin, one-seventh of the total colorimetric reading was due to this reagent blank.

Plasma from dogs which had received no inulin gave readings slightly higher than those obtained with the combined reagents alone. Color-yielding material was present in plasma in concentrations equivalent to 0.7 to 1.4 mg. of inulin per 100 milliliters. In the analytical procedure, as described, this blank was represented by a reading of 2 to 4 divisions on the colorimeter scale. Therefore, in all renal clearance experiments a blood sample was obtained before the injection of inulin. The plasma of this control sample was treated along with the samples drawn after the injection of inulin, thus serving as a combined plasma and reagent blank for calculation of the net amount of inulin in the clearance samples. It is felt that this method of correcting for the plasma blank did not serve as a significant source of possible error, since the correction was of a very low order of magnitude and varied from time to time only within very narrow limits.

The undiluted urine of normal dogs was found to give high "blank" values for inulin. However, the extent to which urine samples from clearance experiments were diluted, in order to reduce the inulin concentration to a level suitable for analysis, was sufficient to obviate entirely the effect of the urinary material. Hence, in the analysis of urine samples from clearance experiments, a reagent blank alone served adequately as a color control.

Plasma and urine samples from three normal human subjects who had received no inulin were also subjected to the analytical procedure. The plasma samples gave blank values equivalent to 0.95, 1.2, and 1.4 mg. per cent of inulin. As in the case of dogs, dilutions of urine samples comparable to those which would be used under clearance conditions gave no blank readings.

RESULTS

Standard Curve.—The relationship between colorimetric reading and inulin concentration was studied over a range from 10 to 120 γ of inulin per tube. The results obtained in five separate experiments are recorded in Table I. In each case the relationship could be expressed graphically as a straight line indicating conformity to the Beer-Lambert law over the range of inulin concentrations used. The readings obtained in Experiments A and B fell on the same straight line as shown in Fig. 1. Those of Experiments C, D, and E, performed some

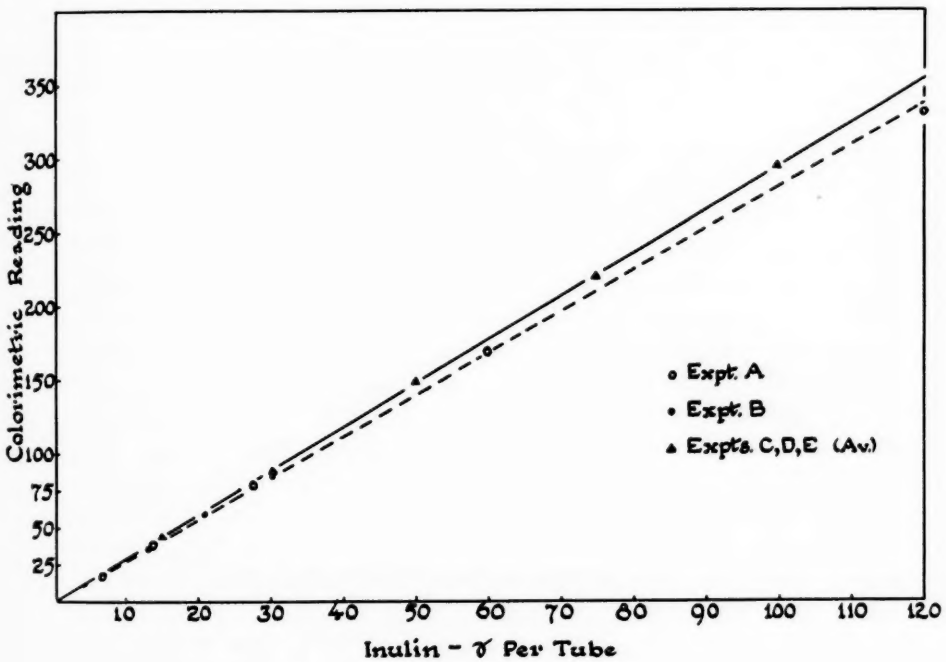

Fig. 1.

months later, also were in very close agreement. The plot of their average likewise yielded a straight line passing through zero, but one which differed slightly from the first as to slope. It appeared, therefore, that all subsequent determinations of the inulin content of unknown material could not be referred to a single standard calibration curve, but that analysis of standard inulin solutions should accompany all determinations of such material. This is illustrated further by the fact that standard tubes containing the same amount of inulin did not yield identical readings on all occasions. Accessory to sixty-six determinations of the inulin content of samples of blood and urine during a period of four months, duplicate tubes of two different concentrations of inulin standard were analyzed. The readings obtained differed by more than two "Klett-points" on only six occasions. The mean reading for the averages of sixty-six sets of duplicates containing 26.7 γ of inulin per tube was 81, with a standard deviation of 4.5 per cent. On twenty-eight similar occasions, when the

TABLE I. THE RELATIONSHIP BETWEEN INULIN CONCENTRATION AND COLORIMETRIC READING ON FIVE DIFFERENT OCCASIONS

INULIN CONCENTRATION (γ PER TUBE)	COLORIMETRIC READING* A (9/29/48)	B (10/21/48)	INULIN CONCENTRATION (γ PER TUBE)	COLORIMETRIC READING* C (4/24/49)	D (4/24/49)	E (4/25/49)	AV.
6.8	19		15	45	44	45	45
13.0		36.5	30	90	88	89	89
13.7	39.0		50	149	150	148	149
21.0		60.0	75	222	223	222	222
27.4	77.5		100	300	295	293	296
30.0		87.0					
59.7	169.0						
119.4	333.0						

*Klett-Summerson colorimeter (filter No. 54); triplicate determinations were done throughout.

inulin concentration was 33.3 γ per tube, the mean colorimetric reading was 95, with a standard deviation of 5.5 per cent. These results show that a single standard calibration curve should not be used and that standards must be included with each set of determinations. On the other hand, duplicates on any single occasion agreed very well. The recovery experiments discussed below, in which as many as ten aliquots from a single filtrate were analyzed simultaneously, show that the spread of values on any one occasion is very small.

Filter No. 54 of the colorimeter was used in all the experiments already discussed. Since the colored reaction product had an absorption maximum at 480 mμ in the Beckman spectrophotometer, it was thought that filters No. 47 and No. 50 of the colorimeter might result in steeper calibration curves. This possibility was tested by reading the tubes with all three filters on several occasions. Neither filter No. 47 nor No. 50 yielded the straight-line relationship which was obtained consistently with No. 54. This discrepancy was not encountered with determinations which were made with the Beckman spectrophotometer at 470, 480, and 540 millimicrons. However, the advantage of speed offered by the colorimeter led to its use with filter No. 54 despite this finding, a procedure which has yielded uniformly satisfactory results.

TABLE II. THE RECOVERY OF ADDED INULIN FROM PLASMA AND FROM URINE WITH THE STANDARD ANALYTICAL PROCEDURE

EXPERIMENT	NUMBER OF FILTRATES	NUMBER OF DETERMINATIONS PER FILTRATE	CONC. OF ADDED INULIN (MG. %) PLASMA	URINE	AVERAGE RECOVERY MG. %	± ST. D.	%	± ST. D.
1	1	2	15		15.3		102	
	1	2	15		15.3		102	
	1	2	15		15.0		100	
	1	2	15		14.9		99.7	
2	1	10	15		15	0.20	100	1.3
3	1	10	33		33.2	0.46	100.6	1.4
4	1	10	33		33.3	0.47	101	1.4
5	1	10	40		41	0.38	102.5	0.9
6	1	10		200	203	3.7	101.5	1.8
7	1	10		500	500	4.0	100	0.8

Recovery Experiments.—For these experiments inulin was added to normal dog plasma and urine in quantities sufficient to produce final concentrations of the order encountered in clearance studies. Analyses for inulin content then were performed as previously described. The consistent and complete recovery of inulin on seven different occasions is shown in Table II.

TABLE III. THE INCONSISTENT RECOVERY OF ADDED INULIN FROM PLASMA AND FROM URINE, USING CADMIUM HYDROXIDE AS A PROTEIN PRECIPITANT AND/OR A REACTION TEMPERATURE OF 79 TO 81° C.

EXPERIMENT	TYPE OF FILTRATE	NUMBER OF FILTRATES	NUMBER OF DETERMINATIONS PER FILTRATE	TEMPERATURE (°C.)	CONC. OF ADDED INULIN (MG. %)		AVERAGE RECOVERY	
					PLASMA	URINE	MG. %	%
1	$Cd(OH)_2$	1	2	79-81	34.2		28.6	83.6
		1	2	"	34.2		38.0	111.11
		1	2	"	34.2		40.0	117.0
		1	2	"	34.2		31.5	92.1
2	$Cd(OH)_2$	1	2	79.0-79.5	15		14.2	94.7
		1	2	"	15		14.2	94.7
		1	2	"	15		14.0	93.3
		1	2	"	15		14.2	94.7
		1	2	"	15		13.4	89.3
3	TCA	2	2	79-81	10		10.9	109
4	TCA	1	2	79-81	10		10.6	106
		1	2	"	10		11.0	110
		1	2	"	10		10.6	106
		1	2	"	10		10.7	107
5	TCA	1	2	79-81	28.6		28.7	100.3
		1	2	"	28.6		29.3	102.4
		1	2	"	28.6		29.0	101.4
		1	2	"	28.6		29.3	102.4
6	TCA	2	2	79-81	57		59.3	104
7	TCA	4	2	79-81		200	208	104
8	TCA	4	2	79-81		500	500	100

The substitution of cadmium hydroxide for trichloracetic acid in the preparation of filtrates led to unsatisfactory results. Extension of the permissible temperature variation from 0.5° C. to 2° C., during the heating period, likewise appeared to have some deleterious effect. Results of experiments in which these departures from the adopted procedure were introduced are recorded in Table III. The detrimental effect of a temperature variation of only 2° C. is illustrated in Table IV. Using a temperature of 79 to 81° C., analysis of aliquots of a plasma or urine filtrate on one occasion (a) often gave a much different result than did analysis of other aliquots of the same filtrate performed several hours later (b) under identical conditions. In some instances the change in values for the plasma and the urine were in the same direction, and of approximately the same magnitude, so that the renal clearances $\frac{UV}{P}$ calculated from the two sets of data agreed fairly well. In other instances, where the second analysis for inulin differed from the first in one direction in the case of the plasma but in the other direction in the case of the urine, the clearance figures calculated from the data differed even more. When the permissible temperature variation was reduced to 0.5° C. (79 to 79.5° C.), the analyses were much more reproducible.

TABLE IV. THE EFFECT OF TEMPERATURE VARIATION DURING COLOR DEVELOPMENT ON THE REPRODUCIBILITY OF RESULTS IN EXPERIMENTS MEASURING THE RENAL CLEARANCE OF INULIN IN DOGS

DOG	SAMPLE	TEMPERATURE (°C.)	INULIN (MG. %) PLASMA	INULIN (MG. %) URINE	$\frac{U}{P}V$ (C.C./MIN.)
1	1	79.0-81.0	(a) 16.4	153	51
			(b) 20.4	205	54.6
1	2	79.0-81.0	(a) 16.7	155	50
			(b) 23.8	212	48.3
1	3	79.0-81.0	(a) 27.8	219	40.6
			(b) 28.2	212	38.6
2	1	79.0-81.0	(a) 18.2	113	58
			(b) 19.3	117	56.4
2	2	79.0-81.0	(a) 16.5	107	60.5
			(b) 18.1	101	52.2
3	1	79.0-79.5	(a) 17.5	209	56.5
			(b) 17.1	205	56.7
3	2	79.0-79.5	(a) 17.5	238	55.8
			(b) 16.9	232	56.3
3	3	79.0-79.5	(a) 17.1	167	53.5
			(b) 16.9	164	53.2
3	4	79.0-79.5	(a) 18.4	168	55.2
			(b) 18.6	171	55.6
3	5	79.0-79.5	(a) 18.1	164	54
			(b) 17.6	164	55.4

(a) and (b), The results of two successive analyses, under identical conditions, of a single filtrate.

It is quite possible that a higher or lower temperature than that used here would have been permissible, as long as the variations were kept within 0.5° C. Calibration curves have been constructed from experiments in which temperatures of 75 to 75.5° C. and 84 to 84.5° C. were used, comparisons being made with the curves obtained at 79 to 79.5° C. The temperature of 79 to 79.5° C. yielded the line with the steepest slope, but in all three instances straight lines, with conformity of all points, were obtained.

Interfering Substances.—The recovery of inulin from aqueous solutions containing other substances used in renal clearance studies also has been studied. Creatinine and sodium *p*-aminohippurate, in concentrations comparable to those encountered in clearance experiments, did not interfere with the quantitative determination of added inulin. Glucose acted as an interfering substance in that it reacted with the reagents to produce additional color. The amount of glucose required to produce a given intensity of color was many times that of inulin. Actual measurements indicated that the color produced by a given amount of glucose in the analytical procedure for inulin was only 0.45 to 0.65 per cent of that produced by an equal amount of the latter substance; i.e., 150 to 240 mg. of glucose were equivalent to 1 mg. of inulin in this respect. It is probable that the plasma blank discussed earlier was due in large part to its glucose content. The effect of glucose does not invalidate the principle of using a single plasma blank, drawn prior to the injection of inulin, in clearance experiments on fasting dogs, because the fluctuation in glucose concentration necessary to produce any significant

change in colorimetric reading is larger than that encountered under such circumstances. On the other hand, clearance experiments in which glucose T_M is to be measured require the maintenance of a glucose concentration in excess of 400 mg. per cent in the plasma. If simultaneous inulin clearances are to be performed in such experiments as a measure of glomerular filtration rate, the additional color produced by glucose in the analytical procedure for inulin becomes a significant factor, especially since the abnormally high glucose content of the plasma cannot be maintained at an entirely constant level. In such experiments the concentration of glucose present in plasma and urine samples was determined first by the Somogyi method.[8] An amount of glucose equal to that found then was added to the inulin standards and the blank values obtained were used in the subsequent calculations.

Inulin and Creatinine Clearance Ratios.—Since the creatinine clearance, as well as the inulin clearance, is regarded as a measure of glomerular filtration in the dog, it naturally was of interest to determine the degree of agreement between the two, as an additional test of the applicability of the foregoing method for inulin determination.

Nine consecutive ten-minute clearance periods were performed in a single dog, following the subcutaneous injection of sufficient inulin and creatinine to produce concentrations of 13 to 18 mg. per cent of each of these in the plasma and the oral administration of sufficient water to produce a copious urine output. The results are presented in Table V. The average ratio of the two clearance values was 1.036, with a standard deviation of 0.032 and a range of individual values from 0.99 to 1.08. In a current study of the nephrotoxic action of a cyanine dye,[9] the simultaneous renal clearance values of inulin and creatinine have been determined in three additional dogs. The average inulin:creatinine clearance ratio for ten control experiments,

TABLE V. THE SIMULTANEOUS RENAL CLEARANCE OF INULIN AND CREATININE IN A DOG DURING NINE CONSECUTIVE TEN-MINUTE PERIODS

(Diuresis was produced by the oral administration of 40 ml. of water per kilogram of weight 1½ hours before the clearances were begun. Weight of dog = 16.0 kg.)

		INULIN		CREATININE		
PERIOD	NET URINE FLOW (ML./MIN.)	PLASMA (MG. %)	CLEARANCE UV / P (ML./MIN.)	PLASMA (MG. %)	CLEARANCE UV / P (ML./MIN.)	CLEARANCE RATIO INULIN : CREATININE
1	4.8	17.6	66	16.4	65.5	1.01
2	4.8	17.3	67	16.0	68	0.99
3	4.3	17.1	60.4	16.4	59.2	1.02
4	4.0	16.5	57.8	16.0	56.7	1.02
5	3.7	15.6	58.3	15.4	57.4	1.02
6	3.7	14.6	61.5	14.9	56.8	1.08
7	2.2	14.4	55.0	14.0	52.2	1.05
8	1.4	13.9	52.5	13.2	50.0	1.05
9	1.2	13.3	51.2	12.8	47.6	1.07
					Average	1.036
					S.D.	.032

performed before injection of dye and consisting in each case of three to five consecutive ten-minute clearance periods, was 1.03 with a standard deviation of 0.06.

SUMMARY

1. A simple colorimetric method for the quantitative determination of inulin in samples of plasma and urine has been described. The procedure, a modification of similar ones described in the literature, is based on the production of an orange-red color by the reaction of hydrolyzed inulin with resorcinol in strong hydrochloric acid, i.e., a modified Seliwanoff reaction. The principal modifications involve the use of trichloracetic acid as a protein precipitant and the maintenance of rigid temperature control during the final step of color development.

2. Data showing the consistent quantitative recovery of added inulin from plasma and urine are presented. The results of a few simultaneous measurements of the renal clearance of inulin and creatinine are described also.

The authors are indebted to Dr. Ernest Bueding and Dr. T. G. Bidder for their valuable advice regarding numerous aspects of this study.

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METHODS OF ISOLATION AND INCIDENCE OF BRUCELLA TYPES FOUND IN GEORGIA

ALICE BRIM, M.S., JANIE F. MORRIS, M.S., AND E. J. SUNKES, M.S., D.P.H.
ATLANTA, GA.

DURING the nine-year period, from 1940 through 1948, the laboratories of the Georgia Department of Public Health isolated brucella organisms from 366 individuals and demonstrated agglutinins to an appreciable titer with a brucella antigen in the blood serum of 1,057. The methods and data recorded here are the results of searching for (1) more efficient techniques in culturing blood for brucella and (2) greater ease in handling a large number of specimens. This report is made chiefly with the view of presenting methods now in use and the gross results obtained by the methods employed during the nine-year period.

METHODS AND MATERIALS

The Agglutination Test.—Whole clotted blood usually is received for the various tests, including blood cultures, agglutination series, and other serologic tests. The information forms accompanying the specimens are studied carefully for the physician's requests, his findings, and tentative diagnosis. Serum for demonstrating brucella agglutinins is removed aseptically, leaving the clot uncontaminated.

The macroscopic tube agglutination test is performed using a *Brucella abortus* antigen prepared from *Br. abortus,* No. 456. The culture, which was obtained from the National Institute of Health, is checked for smoothness each time the antigen is prepared. Forty-eight hour growth of the organism on tryptose agar[3] is harvested in 0.5 per cent phenolized saline solution, heated at 60 to 65° C. for thirty minutes, and checked for sterility. The antigen is then centrifuged, washed with fresh saline, and diluted to correspond with barium sulfate standard No. 2. Each lot of antigen is tested with serums of high, low, and negative titers.

The patient's serum is diluted in 0.5 ml. amounts with 0.85 per cent sodium chloride using the twofold progression beginning with a 1:20 dilution through 1:640. Five-tenths milliliter of antigen is added to each tube. The tests are incubated four to six hours in a 45° C. water bath and refrigerated overnight before reading. Though this laboratory is aware of the problem in reporting certain findings in the agglutination test as "positive" or being of "diagnostic titer," a test showing moderate to complete agglutination in the dilution of 1:320 or higher is considered "positive." Slight to moderate agglutination in dilutions lower than 1:320 is considered "doubtful" and a second specimen is requested to demonstrate a rise in titer.

Blood Clot Cultures.—The blood clots are cultured for brucella under any one of the following conditions:

(1) If requested by the physician.

(2) If a "positive" or "doubtful" agglutination reaction as already described is obtained.

(3) If the clinical evidence presented by the physician strongly indicates brucellosis although agglutinins are not demonstrated.

Prior to June, 1947, all cultures for brucella were incubated at 37° C. in candle jars. Since then they have been grown in a special CO_2 incubator devised in the Atlanta laboratory

From the Georgia Department of Public Health Laboratories.
Presented before the Laboratory Section of the Southeastern Branch of the American Public Health Association at the Annual Meeting in Dallas, Texas, April 15, 1949.
Received for publicaton, Nov. 12, 1949.

in the Georgia Department of Public Health.[4] The incubator maintains an average atmosphere of approximately 10 per cent CO_2 and has proved satisfactory for growing brucella as well as other bacteria.

Until July, 1948, the clot was comminuted[1] through a sterile 10 ml. syringe without a needle into a bottle containing about 30 ml. of tryptose broth.[2] The bottles were incubated four weeks in candle jars and subcultured weekly to tryptose agar slants.[3] If contaminants were present, tryptose agar plates containing 1:700,000 crystal violet were employed. Subcultures were examined after forty-eight and seventy-two hours, and if negative, were held a week before final examination.

Since July, 1948, we have used a culture bottle described by Castaneda[5] containing both the broth for enriching the bacteria and the agar for the subculture. About 15 ml. of tryptose agar are sterilized in a 4 ounce drug bottle closed with a gauze-covered cotton plug. While the agar is still liquid, the bottle is laid flat side down to allow solidification of the agar on the broad wall. Sterile tryptose broth is added and the bottle incubated for sterility before using.

After the clot is mashed through the syringe into the bottle it is incubated in the upright position in the CO_2 incubator. Twice weekly the bottles are tilted carefully so that the blood-broth mixture spreads over the agar, thus making the subculture. Incubation is continued for a month unless brucella organisms are isolated earlier. If contaminants are present, subcultures are made to crystal violet tryptose agar plates which are held one week before being finally examined.

When suspicious growth is obtained, a Gram stain is made and a slide agglutination test is done using *Br. abortus* antiserum. Further identification and typing is effected by planting the cultures on tryptose agar plates containing appropriate dilutions of thionin and basic fuchsin.[6] In the case of atypical cultures, investigation of nitrate reduction and CO_2 requirements is made. Hydrogen sulfide production tests have not been particularly helpful.

Special Blood Culture Outfits.—When agglutinins are demonstrated as high as 1:320 dilution of the patient's serum, a special blood culture bottle is mailed to the physician. This outfit consists of a rubber-stoppered, flint glass, vaccine bottle containing about 30 ml. of tryptose broth with sodium citrate[2] and 10 per cent carbon dioxide[7] The bottle carries a label directing the physician to swab off the rubber stopper with iodine and to inoculate through the stopper 5 to 10 ml. of blood collected aseptically.

When the outfit is returned to the laboratory it is incubated and subcultured to tryptose agar at weekly intervals for four weeks. Subcultures are obtained by withdrawing through the stopper a drop of culture with a sterile hypodermic needle and syringe. Tryptose agar with or without crystal violet is used for planting, depending on the presence or absence of contaminants. Subsequent growth is examined and identified as previously described.

Attempts at adapting the Castaneda method for the mailed outfits were not wholly successful. Because of rough handling in the mails, the agar would occasionally break away from the walls of the bottle, thus making it necessary to subculture to demonstrate bacterial growth.

RESULTS

During the period from 1940 through 1948, the blood serum of 1,057 individuals showed moderate to complete agglutination in the 1:320 or higher dilutions with brucella antigen. Brucella organisms were isolated from 454 blood cultures on 366 individuals. Of these isolates, 345 were *Br. suis* and 108 were *Br. abortus.* As shown in Table I, 273 (74.6 per cent) of the patients were infected with *Br. suis* and 92 (25.1 per cent) with *Br. abortus;* one culture was not viable when typing was attempted. Because of the fact that the physicians sometimes submitted several specimens of clotted blood from a patient,

TABLE I. INCIDENCE OF BRUCELLA TYPES ISOLATED IN GEORGIA (1940-1948)

YEAR	POSITIVE BLOOD CULTURES	BR. SUIS	BR. ABORTUS
1940	22* (20)†	20 (18)	2 (2)
1941	42 (32)	34 (26)	8 (6)
1942	38 (35)	31 (28)	7 (7)
1943	30 (26)	20 (16)	10 (10)
1944	54 (43)	39 (32)	15 (11)
1945	62 (54)	42 (36)	20 (18)
1946	58 (47)	42 (33)	16 (14)
1947	67 (53)‡	49 (38)	17 (14)
1948	81 (56)	68 (46)	13 (10)
Total	454 (366)	345 (273)	108 (92)

*Isolations.
†Individuals.
‡One untyped (strain not viable when typing was attempted).

the chances for isolating the organism were multiplied. Possibility of obtaining a positive culture was further enhanced by the use of the special outfits sent to the physicians for inoculation with the whole blood.

Approximately 3,600 blood specimens including clots and whole blood were cultured for brucella during the period under consideration. Though many attempts were made, no isolations were obtained where the serum gave a negative or a weak reaction (slight agglutination in low dilutions) with brucella antigen. These findings are in general agreement with those of the epidemiologic study of brucellosis in Minnesota[8] in which only one patient failed to show agglutinins in a series of 268 from whom brucella was recovered.

The preponderance of the *suis* type parallels the findings in other states where diversified farming, cattle and hog raising and slaughtering, and meat packing are outstanding occupations of the rural and some of the urban populations.[9] The prevalence of the brucella infections caused by drinking raw milk from *suis*-infected cows is not known. However, in 1930, Atwood and Hasseltine[10] reported eleven cases of brucellosis in Ware County, Georgia, with evidence indicating that they were due to *Br. suis* from an infected milk supply. It is notable that the Minnesota workers[8] found a relatively low incidence of *Br. suis* infection even in the section of the state where many hogs are raised.

Of the 454 isolations of brucella there were five which did not grow on tryptose agar containing thionin and fuchsin either in the dilutions used for routine typing or in lower concentrations. These strains were tentatively considered to be *Br. abortus*. Four strains were isolated which grew on both dyes, produced hydrogen sulfide to a degree similar to *suis*, and gave a nitrate reduction reaction resembling that of *suis*. Dr. I. F. Huddleson's laboratory confirmed the tentative identification of these nine cultures.

Fig. 1 shows the geographic distribution of the brucella types isolated by the laboratories of the Georgia Department of Public Health during the nine-year period. The type of brucella isolated from man in certain areas of the state corresponds in general with the type of animal industry practiced

there. The swine industry predominates in the southern half. Several large abattoirs devoted chiefly to processing pork products are located in that area. Brucella infection in the swine herds is known to be quite extensive and the type of organism isolated from human beings is correspondingly *suis*. Dairy-

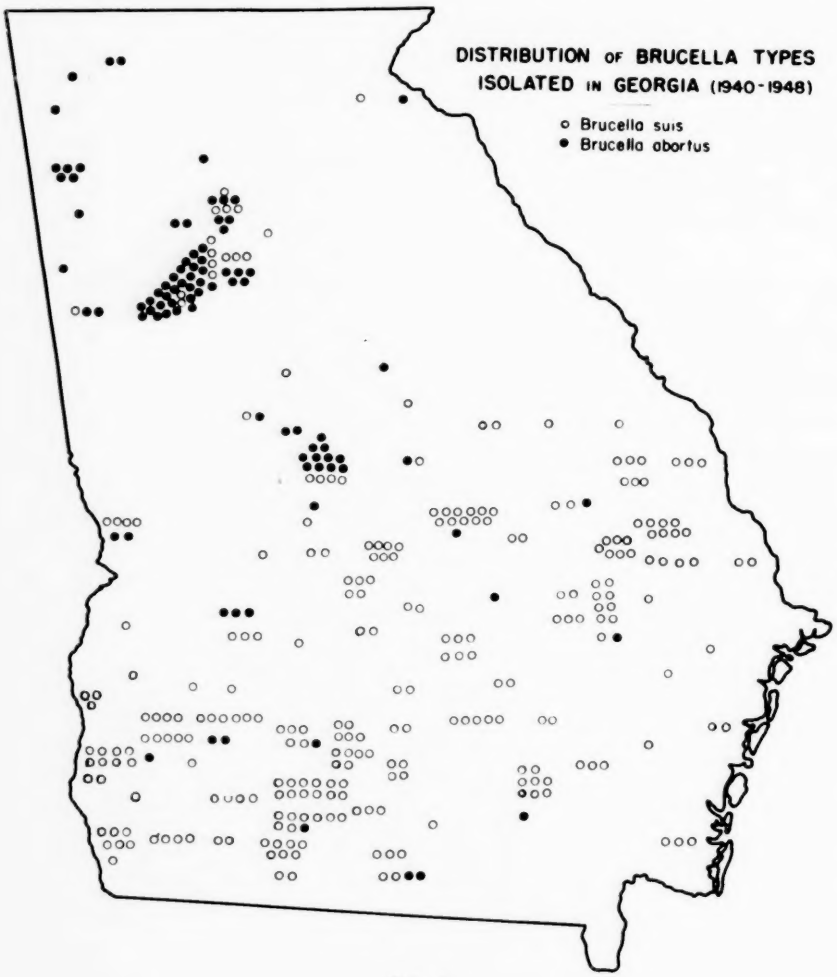

Fig. 1.

ing is the prevalent agricultural industry in the northern section, this area serving as the milkshed for the larger cities. Isolations from this area are chiefly the *abortus* type.

SUMMARY

1. A general description is given of the methods used in the agglutination test for brucellosis and in the culturing of blood specimens for brucella.

2. During the nine-year period (1940-1948) the serums of 1,057 individuals showed moderate to complete agglutination with brucella antigen as high as a 1:320 dilution or above. Approximately 3,600 blood specimens were exam-

ined for brucella organisms. There were 454 brucella isolations representing 366 individuals, all of whom showed serum agglutinins in a titer of 1:320 or above. There were 345 isolations of *Br. suis* from 273 patients and 108 *Br. abortus* isolations from 92 patients. Though numerous attempts were made, no isolations were obtained from specimens on which the agglutination test was negative or which showed slight agglutination in low dilutions.

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THE DETERMINATION OF SENSITIVITY TO PENICILLIN AND STREPTOMYCIN OF ENTEROCOCCI AND STREPTOCOCCI OF THE VIRIDANS GROUP

E. Jawetz, M.D., and J. B. Gunnison, M.A.
San Francisco, Calif.

THE clinical laboratory is often called upon to test bacteria isolated from patients for sensitivity to antibiotics. The request is made most frequently when the bacteria were recovered from the blood stream, such as in cases of bacterial endocarditis and other stubborn and relatively inaccessible infections. The result of the in vitro sensitivity test often serves as a basis for the choice of drug and dosage administered to the patient.

In the majority of streptococcal infections the results of penicillin sensitivity tests performed on freshly isolated strains have shown good correlation with the observed clinical response to penicillin therapy.[2, 4] Notable exceptions were reported in some infections due to enterococci[1, 7] or to *Streptococcus sanguis*.[10] To cure such infections it has often been necessary to employ amounts of penicillin far in excess of the dosage estimated on the basis of in vitro sensitivity tests. In other instances, penicillin alone has been unsuccessful but combinations of this drug with streptomycin eliminated the infection.[7, 11]

Inadequate chemotherapy not only may delay the patient's recovery but also may permit serious organic damage and occasionally the development of resistant bacterial variants. If the results of a laboratory test are to serve as a basis for the choice of drug and dosage, the test must be reliable and reproducible and the interpretation of results must be uniform and clear. The work presented here concerns the apparent discrepancies between the results of penicillin sensitivity tests and therapeutic responses observed with strains of enterococci, streptococci of the viridans groups, and *Str. sanguis*.

MATERIALS AND METHODS

Streptococcus Cultures.—The following strains were included in the observations reported here.

Streptococci of the Viridans Group: Seven strains isolated from cases of subacute bacterial endocarditis or the upper respiratory tract of patients.

Streptococcus sanguis[10]: Three strains labeled s.b.e. 21, s.b.e. 90, and s.b.e. 95 (kindly made available to us by Dr. E. Alture-Werber). These strains exhibited the characteristics outlined by the original investigators.[10]

From the Division of Bacteriology, University of California Medical School.

Supported in part by a grant from the Research Committee, University of California, Medical School.

The streptococcal group names are used in this paper as defined by Sherman (J. Bact. 35: 81, 1938). The term "enterococci" includes the species *Str. faecalis*, *Str. liquefaciens*, *Str. zymogenes*, and *Str. durans*. The group characteristics are given under Methods. The term "viridans group" includes the species *Str. salivarius*, *Str. bovis*, *Str. equinus*, and *Str. thermophilus*. Because of the essential similarity of the species within the groups, no attempt was made to classify them here.

Received for publication, Nov. 15, 1949.

Enterococci: Eighteen strains (five of them kindly supplied by Dr. L. A. Rantz) isolated from the blood stream or the urinary tract of patients in San Francisco. The enterococci were typical morphologically and culturally. All belonged to Lancefield Group D. They grew freely in broth containing 6.5 per cent sodium chloride or 0.1 per cent methylene blue. They multiplied at pH 9.6. None of the other streptococci tested had these characteristics.

Culture Methods.—All stock cultures were kept in 10 per cent blood broth at 4° C. for the duration of the experiments. The bacteria were grown in, and the sensitivity tests were carried out in Proteose Peptone #3 (Difco) broth of the following composition:

Proteose Peptone #3	20 Gm.
Bacto-Dextrose	0.5 Gm.
Sodium chloride	5 Gm.
Disodium phosphate	5 Gm.
Water	1,000 ml.

Subcultures were made on agar plates having the same base. All cultures were incubated at 37° C.

Sensitivity Test.—The sensitivity tests were set up in small, metal-capped culture tubes in a total volume of 2 milliliters. Commercial crystalline sodium penicillin G (60 mg. = 100,000 units) and commercial streptomycin sulfate were dissolved in sterile physiologic salt solution. The desired final concentrations were prepared in broth. Fresh solutions were used in every test. The inoculum of bacteria was 0.1 ml. of an eighteen-hour broth culture containing 10^8 to 10^9 organisms per milliliter (i.e., groups or chains of streptococci).

The inoculated tubes were incubated at 37° C. for twenty-eight days and inspected daily for the development of turbidity. At regular intervals a standard loopful (0.005 ml.) of broth from each tube was streaked on a segment of an agar plate. These subcultures were incubated at 37° C. for three days and the number of colonies per segment was counted and tabulated as follows:

C = Confluent growth on plate, turbidity in broth tube.
++++ = More than 100 colonies on plate, no turbidity in broth tube.
+++ = 50 to 100 colonies on plate, no turbidity in broth tube.
++ = 10 to 50 colonies on plate, no turbidity in broth tube.
+ = 1 to 10 colonies on plate, no turbidity in broth tube.
− = No colonies on plate (i.e., negative subculture), no turbidity in broth tube.

A negative subculture was believed to indicate that the total number of viable bacteria in the broth tube was less than 200. When larger volumes of broth from such tubes were subcultured in liquid media, organisms could sometimes be recovered. At the end of twenty-eight days, or occasionally earlier, 0.1 ml. of commercial penicillinase (Bacto-Penase) was added to all tubes which had remained clear up to that time, and the subsequent appearance of turbidity or of growth in subculture was observed. This amount of penicillinase is said to inactivate about 1,000 units of penicillin. Tubes showing no growth in the six days following the addition of penicillinase were considered sterile.

RESULTS

Effect of Size of Bacterial Inoculum on Results of Sensitivity Test.—Any bacterial population is a heterogenous collection of organisms of different characteristics including different susceptibilities to antibiotic agents. A rigorous test should detect the more resistant organisms rather than the average sensitivity of the group. Spicer and Blitz[13] have summarized the arguments in favor of maximal rather than minimal inocula in antibiotic sensitivity tests. Table I illus-

trates the effect of bacterial inocula of different sizes on the results of penicillin sensitivity tests with enterococci. All studies reported below were carried out with inocula of 10^7 to 10^8 organisms.

TABLE I. RELATIONSHIP OF SIZE OF INOCULUM TO RESULTS OF PENICILLIN SENSITIVITY TESTS WITH ENTEROCOCCI

INOCULUM NUMBER OF ORGANISMS	TUBES CONTAINING PENICILLIN (UNIT/ML.)							
	1	2.5	5	10	25	50	100	500
10^7	1*	1	2	3	5	5	5	5
10^5	1	3	5	6	–	–	–	–
10^3	1	5	15	–	–	–	–	–

–, No turbidity for twenty-eight days.
*Day of appearance of turbidity in broth tube, when incubated at 37° C.

Inhibition vs. Death of Bacteria Exposed to the Action of Penicillin.—The bactericidal nature of penicillin action in vitro was acknowledged early[3] but it was also recognized that organisms might survive in assay tubes showing no turbidity after incubation and might be detected only by subculture. The usual report states that, "the organism was inhibited by . . . units penicillin per milliliter" or ". . . was sensitive to . . . units penicillin per milliliter," taking as end point the lack of grossly visible growth in or on the medium. This end point may indicate rapid death of all inoculated bacteria in some species; in others, it might be compatible with survival of many bacteria. The meaning of this end point would obviously not be the same in these two situations. Tests were made to determine whether such differences might occur with different streptococci.

Large inocula (10^7) were added to various concentrations of penicillin in broth and subcultures were made frequently in the course of incubation at 37° C. Representative results obtained with strains of the viridans group, enterococci, and *Str. sanguis* are shown in Table II. The viridans streptococci all failed to

TABLE II. EFFECT OF INCREASING CONCENTRATIONS OF PENICILLIN ON THE SURVIVAL OF CERTAIN STREPTOCOCCI

	DAY SUBCULTURED	RESULT OF SUBCULTURE FROM TUBES ORIGINALLY CONTAINING PENICILLIN (UNIT/ML.)									
		0	0.1	1.0	5.0	10.0	25.0	50.0	100.0	250.0	500.0
Viridans streptococcus, strain 15	2	C	–	–	–	–	–	–	–	–	–
	5	C	–	–	–	–	–	–	–	–	–
	7	C	–	–	–	–	–	–	–	–	–
Enterococcus, strain 3	2	C	C	C	C	++++	++++	++++	++++	+++	++++
	5	C	C	C	C	++++	+	+	+	++++	++++
	7	C	C	C	C	C	++	–	–	++	+
	14	C	C	C	C	C	+	+	–	–	+
Str. sanguis, strain s.b.e. 95	2	C	++	+	–	–	–	–	–	–	–
	5	C	+	–	–	–	–	–	–	–	–
	7	C	–	–	–	–	–	–	–	–	–

C, Confluent growth on agar plate, broth tube turbid.
++++, More than 100 colonies on agar plate, broth tube clear.
+++, 51 to 100 colonies on agar plate, broth tube clear.
++, 10 to 50 colonies on agar plate, broth tube clear.
+, 1 to 10 colonies on agar plate, broth tube clear.
–, No colonies on agar plate, broth tube clear.

produce turbidity in broth in 0.1 unit per milliliter penicillin and no organisms survived in that concentration for even twenty-four hours. Two of three strains of *Str. sanguis* produced no turbidity in broth containing 0.1 to 1.0 unit per milliliter penicillin but survived for three days in these concentrations.

The eighteen strains of enterococci studied all behaved similarly. After forty-eight hours of incubation there was turbidity in broth with penicillin concentrations up to 2.5 or 5.0 units per milliliter. The next tube in the series, containing a higher concentration of penicillin, remained clear. In the usual test, that concentration would be reported as the "sensitivity" of the organism. Subculture on solid media, however, made it evident that significant numbers of organisms survived for five to seven days in initial penicillin concentrations of 500 or 1,000 units per milliliter. As incubation continued, additional tubes originally containing 10 to 50 units per milliliter of penicillin became turbid, while in higher concentrations of the antibiotic the number of bacteria gradually declined. On the fourteenth to twenty-eighth day of incubation some tubes were turbid, yielding confluent growth in subculture; the remainder were clear and sterile. At that time the penicillin originally present had deteriorated markedly so that only a small fraction of its original activity remained.

The Zone Phenomenon in Penicillin Sensitivity Determinations.—It has been observed by Kirby,[9] Eagle,[5] and others that with some bacteria the rate of bactericidal action of penicillin increases with the concentration of the antibiotic up to an optimum, then declines sharply with higher concentrations. This "paradoxical zone phenomenon" was observed particularly with some strains of staphylococci and enterococci. It permitted the definition of a narrow optimal range of effective concentrations for the antibiotic.

A zone phenomenon of penicillin activity was encountered in this study with many strains of enterococci. It manifested itself in two ways:

(a) Sometimes certain penicillin concentrations, e.g. 25, 50, or 100 units per milliliter, rapidly killed the entire inoculum, whereas organisms persisted in both higher and lower concentrations for long periods (Table III).

(b) In some instances, as higher penicillin concentrations, e.g. 100 or 1,000 units per milliliter, deteriorated in the course of prolonged incubation at 37° C., the bacterial population slowly diminished to low levels. Subsequently either all

TABLE III. ZONE PHENOMENON IN THE ACTION OF PENICILLIN ON SOME STRAINS OF ENTEROCOCCUS

ENTEROCOCCUS STRAIN	DAY SUBCULTURED	RESULT OF SUBCULTURE FROM TUBES ORIGINALLY CONTAINING PENICILLIN (UNIT/ML.)							
		1	5	10	25	50	100	250	500
16	2	C	C	−	+	−	−	+++	+++
	5	C	C	C	−	−	−	++	++
	7	C	C	C	++	+	−	+	+
	14	C	C	C	C	−	−	−	−
17	2	C	C	++++	++++	++++	++++	++++	++++
	5	C	C	C	−	+	++	+++	+++
	7	C	C	C	+	−	+	+	+
	14	C	C	C	C	C	−	−	−

Symbols are the same as in Table II.

organisms died or the survivors suddenly multiplied to produce turbidity. These changes occurred first in the lowest and last in the highest concentrations of penicillin. Possibly a high original penicillin concentration, outside of the optimal zone, and with lessened bactericidal action, may deteriorate until it reaches its effective zone and destroys most or all viable bacteria. Further deterioration may reduce the levels of penicillin below the effective zone permitting any surviving organisms to multiply rapidly.

Combined Penicillin-Streptomycin Assay.—Because of the frequent resistance of enterococcal infections to massive doses of penicillin, combined penicillin-streptomycin therapy has been attempted in some cases with good results.[7, 11] A laboratory test for the estimation of dosage in such combined therapy would be helpful.

Comparative studies were carried out to determine the effect of a constant amount of streptomycin added to the penicillin in sensitivity tests. Representative results with two strains of enterococci are shown in Table IV. With massive inocula, enterococci were not inhibited by streptomycin alone in concentrations

TABLE IV. EFFECT OF PENICILLIN-STREPTOMYCIN MIXTURES ON ENTEROCOCCI

ENTEROCOCCUS STRAIN	STREPTOMYCIN (μG/ML.)	DAY SUBCULTURED	RESULT OF SUBCULTURE FROM TUBES ORIGINALLY CONTAINING PENICILLIN (UNIT/ML.)							
			0	1	2.5	5	10	50	100	500
16	0	2	C	C	C	C	–	+	–	+++
		5	C	C	C	C	C	–	–	++
		7	C	C	C	C	C	+	–	+
		14	C	C	C	C	C	–	–	–
	50	2	C	C	++	+	+	+	–	–
		5	C	C	+	–	–	–	–	–
		7	C	C	+	–	–	–	–	–
		14	C	C	–	–	–	–	–	–
17	0	2	C	C	C	C	++++	++++	++++	+++
		5	C	C	C	C	C	+	++	++
		7	C	C	C	C	C	–	+	+
		14	C	C	C	C	C	C	–	–
	50	2	C	C	C	C	+++	++	+++	++
		5	C	C	C	C	C	+	++++	–
		7	C	C	C	C	C	–	–	–
		14	C	C	C	C	C	–	–	–

Symbols are the same as in Table II.

of 250 μg per milliliter or less. Yet the addition of 25 or 50 μg per milliliter (concentrations that may be achieved in body fluids with therapeutic doses) markedly modified the effect of penicillin. The effectiveness of penicillin was increased from ten to several hundred fold. Furthermore there were no viable organisms demonstrable in the higher penicillin concentrations, i.e. the zone phenomenon did not occur. There appeared to be a true synergistic effect of the two antibiotics on enterococci. A more detailed analysis of this phenomenon will be presented elsewhere.[6]

A test for the combined effect of penicillin and streptomycin might prove useful in evaluating the resistance of organisms before using multiple antibiotic therapy. The evaluation must take into account both (a) the increase in inhibition, i.e. bacteriostatic effect, as measured by the lack of visible growth and (b) the rapid and complete elimination of viable bacteria from tubes without visible growth, i.e. increase in bactericidal effect. This is illustrated in Table IV. Strain 16, from a patient whose subacute bacterial endocarditis *(Str. faecalis)* was cured by 5,000,000 units of penicillin and 2 Gm. of dihydrostreptomycin daily for four weeks,* is contrasted with strain 17 from a similar patient who was not cured by 4,000,000 units of penicillin and 2 Gm. of dihydrostreptomycin daily for four weeks.[8] In strain 16, the addition of 50 μg per milliliter streptomycin had eliminated all viable organisms from broth containing 5.0 units per milliliter of penicillin in five days. At the same time the mixture of 50 μg per milliliter streptomycin with 250 units per milliliter penicillin had not killed all organisms of strain 17. In other tests with the same strains, much smaller differences were observed. Therefore it is doubtful whether such a test can distinguish between strains with respect to their response to combined therapy. Further experience will be needed to establish this point.

To establish the presence of adequate blood levels early in combined antibiotic therapy a modification of the test proposed by Schlichter and co-workers[12] has given satisfactory results. In this test, dilutions of the serum freshly obtained from the patient under treatment are mixed with suspensions of the infecting organism. The ability of the serum to inhibit the bacteria in high dilution is taken as direct evidence of satisfactory drug levels in the blood stream. The reservations concerning inhibitory vs. bactericidal properties of antibiotics discussed previously likewise apply to this test. In this laboratory, subcultures from serum-bacteria mixtures have been used to detect viable organisms surviving in tubes showing no gross turbidity, and only the end point of bactericidal action has been considered.

DISCUSSION

Results obtained in the test tube cannot be applied to human disease. However, when in vitro results agree with clinical observations, some inferences as to possible mechanisms of action may be permissible. Bacterial endocarditis due to enterococci has frequently been resistant to penicillin therapy even when the dosage was commensurate with the apparent resistance of the organisms in vitro. Such cases usually have negative blood cultures during the administration of penicillin but the organisms reappear when the drug is stopped. The bacteria have been suppressed, not killed.

These clinical observations are consistent with the finding that a number of enterococci survive for many days at 37° C. in broth containing large amounts of penicillin. These cocci do not produce visible growth until the penicillin is

*Dr. L. A. Rantz kindly informed us of the case and made the culture available.

either inactivated by adding penicillinase or has deteriorated to levels that are no longer inhibitory. However, these surviving organisms are not more resistant to penicillin action than the original culture.[6]

Enterococci behave in vitro quite differently from viridans streptococci. With the viridans group, absence of visible growth in media containing penicillin means actual death of the organisms. With the enterococci, a similar "end point" appears to be meaningless. Among the few strains of *Str. sanguis* examined, two behaved somewhat like enterococci except that in the absence of visible growth survivors were found only in low concentrations (0.1 to 1.0 units per milliliter) of penicillin. This agrees with the clinical experience[10] that endocarditis due to *Str. sanguis* does not respond to doses estimated on the basis of apparent inhibition in the test tube, but appears to be cured frequently by administration of larger doses of 1 to 2 million units of penicillin daily. Such large doses could be expected to result in truly bactericidal levels of penicillin for *Str. sanguis* strains. This streptococcal variety appears to occupy a position intermediate to the usual viridans group and the enterococci, both in vivo and in vitro.

The laboratory observations described seem important from the standpoint of the clinical laboratory called upon to guide chemotherapy with sensitivity tests on microorganisms isolated from patients. Bacteria of clinically resistant varieties (e.g. staphylococci and enterococci) should be examined with the following points in mind: (a) The need for a large inoculum providing an adequate sample of the bacterial population. (b) The use of a tube dilution method in which tubes remaining clear are subcultured after forty-eight to ninety-six hours of incubation to detect organisms inhibited, but not killed, by the antibiotic. (c) The presence of viable bacteria in high concentrations of penicillin after incubation for forty-eight hours. This is presumptive evidence that either exceedingly high doses of penicillin or combined chemotherapy should be used.

The "paradoxical zone phenomenon" is important in the interpretation of the test and throws some light on the mechanism of drug action in vitro. Its significance in clinical practice is unknown. It has been suggested[5] that some infections might respond better to low than to very high penicillin doses. No cases have been reported to date in which the patient recovered with small amounts of penicillin after failing to recover with a high dosage regimen. Evaluation of the clinical significance of this phenomenon will be very difficult because of the constant shift of drug levels in the body.

It appears likely that a drug must be actually bactericidal, not only inhibitory, to cure bacterial endocarditis and perhaps other, chronic, low-grade inflammatory processes. Sulfonamides inhibit viridans streptococci but practically never cure subacute bacterial endocarditis. Aureomycin effectively inhibits many enterococci in vitro but does not kill them[6] and rarely results in clinical cure.[8] Penicillin is exceedingly effective in subacute bacterial endocarditis due to viridans streptococci, perhaps because it is rapidly bactericidal for most of these organisms. Its common failure in enterococcal endocarditis might be understood in the light of the in vitro results presented here. Similarly the ob-

served rapid bactericidal effect of penicillin-streptomycin mixtures on some enterococci might help to explain the clinical successes. Further tests are needed regarding the possible usefulness of combined antibiotic sensitivity determinations as guides for combined chemotherapy.

SUMMARY

1. Penicillin sensitivity tests were performed on seven strains of viridans streptococci, eighteen strains of enterococci, and three strains of *Str. sanguis*, using a tube dilution technique with frequent subcultures on solid media.

2. With streptococci of the viridans group, the absence of visible growth after forty-eight hours indicates that all bacteria in the tube have been killed.

3. With enterococci, viable organisms are found in broth containing high concentrations of penicillin after many days of incubation at 37° C. The absence of visible growth does not indicate bactericidal but merely bacteriostatic action of the antibiotic.

4. Two of three strains of *Str. sanguis* occupied an intermediate position between enterococci and viridans streptococci with respect to penicillin sensitivity. They survived for a number of days in low (0.1 to 1.0 units per milliliter) but not in high (2 to 10 units per milliliter) concentrations of penicillin.

5. A zone phenomenon was frequently observed in sensitivity tests with enterococci. A certain penicillin concentration resulted in death of all organisms, whereas both higher and lower concentrations of the antibiotic permitted survival of enterococci for many days. No zone phenomenon was observed with the other streptococci.

6. The addition of streptomycin to penicillin resulted in synergistic action of the two antibiotics on enterococci. When combined, relatively small amounts of both drugs had a bactericidal effect.

7. The possible relationships between the in vitro observations and clinical findings are discussed.

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ERRATUM

In the paper by Landowne, Thompson, Jr., and Ruby, "The Minimal Sodium Diet: A Controlled Study of Its Effect Upon the Blood Pressure of Ambulatory Hypertensive Subjects," in the October 1949 issue of the JOURNAL, the senior author inadvertently omitted an important footnote from the final manuscript revision, to be added on p. 1381:

Sodium and creatinine were determined by spectrophotometric colorimeter. A magnesium uranyl acetate method was used for sodium[14] following the modification by C. S. Farmer, suggested to us by E. Farnsworth and J. S. Krakusin of Northwestern University. For creatinine, Folin's method[15] was adapted. The most excellent technical assistance of Miss Ann David is acknowledged.